SURFACE PATTERNING OF POLY(ε-CAPROLACTONE) SCAFFOLDS BY ELECTROSPINNING FOR CELL GUIDANCE

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
ATILIM UNIVERSITY

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
THE DEPARTMENT OF METALLURGICAL AND MATERIALS ENGINEERING

JULY 2020

Approval of the Graduate School of Natural and Applied Sciences, Atilim University.

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ABSTRACT

SURFACE PATTERNING OF POLY(ε-CAPROLACTONE) SCAFFOLDS BY ELECTROSPINNING FOR CELL GUIDANCE

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July 2020, 75 pages

The aim of this work was to produce three dimentional fibrous surface patterns of Poly(ε-Caprolactone) (PCL), onto 2D smooth solvent cast PCL surfaces with electrospinning method by using a mask/stencil for cell guidance. The characterizations of produced scaffolds were done by thickness measurements, Scanning Electron Microscopy (SEM) analyses, contact angle (CA) measurements, Fourier-transform infrared spectroscopy (FTIR) and mechanical tests. According to SEM micrographs, all of the electrospun scaffold surfaces were exhibited bead-free and uniform morphology while solvent cast surfaces were smooth and nonporous. CA measurements revealed that the solvent cast surfaces had moderate hydrophilicity (~60°) while electrospun regions had more hydrophobic character (~110° for fully electrospun surface and ~85° for electrospun patterns). Mechanical testing was showed the produced scaffolds had brittle character. Also cell culture studies were performed with mouse fibroblast (L929) cells for 7 days period and cell attachment assay, MTT assay, flourescence and SEM analyses were done. Cell culture studies were indicated that the solvent cast and electrospun parts have different characteristics for cell behaviour. Thus, it is possible to achieve cell guidance for by manupulating the cell

attachment and proliferation by introducing electrospun patterns onto solvent cast surfaces.
Key words: Cell Guidance, Poly(ε-Caprolactone), Electrospinning, Solvent Casting, Surface Patterns

ÖZ

POLİ(ε-KAPROLAKTON) DOKU İSKELE YÜZEYLERİNİN HÜCRE YÖNLENDİRMESİ İÇİN ELEKTROEĞİRME İLE DESENLENMESİ

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Temmuz 2020, 75 sayfa

Bu çalışmanın amacı, hücreleri yönlendirmek için, bir maske/şablon kullanarak 2D pürüzsüz çözücü döküm yöntemiyle üretilmiş Poli(ε-Kaprolakton) (PCL) yüzeyleri üzerine elektroeğirme yöntemiyle üç boyutlu lifli PCL yüzey desenleri üretmektir. Üretilen doku iskelelerinin karakterizasyonları kalınlık ölçümleri, Taramalı Elektron Mikroskopisi (SEM) analizleri, temas açısı (CA) ölçümleri, Fourier dönüşümü kızılötesi spektroskopisi (FTIR) ve mekanik testlerle yapılmıştır. SEM mikrograflarına göre, tüm elektroeğrilmiş doku iskele yüzeyleri eşdağılımlı düzgün bir morfoloji sergilerken, çözücü döküm yüzeyleri pürüzsüz ve gözeneksizdir. CA (^{o}) ölçümleri, çözücü döküm yüzeylerinin orta derecede hidrofilikliğe (~60°) sahip olduğunu gösterirken, elektroeğrilmiş bölgeler daha hidrofobik bir karaktere sahiptir (elekroeğirme kaplı yüzey için ~110° ve elektroeğrilmiş desenler için ~85°). Mekanik testler, üretilen iskelelerin kırılgan ve gevrek bir karaktere sahip olduğunu göstermiştir. Ayrıca 7 gün süre ile fare fibroblast (L929) hücreleri ile hücre kültürü çalışmaları yapılmış ve hücre tutunma analizi, MTT analizi, floresans ve SEM analizleri yürütülmüştür. Hücre kültürü çalışmaları, hücrelerin, çözücü döküm ve elektroeğrilmiş yüzeylerinde farklı tutunma ve üreme eğilimlerine sahip olduğunu göstermiştir. Böylece, çözücü dökülmüş yüzeyler üzerinde elektroeğrilmiş desenler oluşturularak, hücre tutunması ve proliferasyonunun oluşturulan desenler aracılığıyla manipüle edilmesiyle, hücreleri yönlendirmek mümkün olmuştur.

Anahtar kelimeler: Hücre Yönlendirmesi, Poli(ε-Kaprolakton)(PCL), Elektroeğirme, Çözücü Döküm, Yüzey Desenleri

To all victims of COVID-19…

ACKNOWLEDGMENTS

First of all, I would like to send my deepest regards to my advisor Prof. Dr. Hilal Turkoglu Sasmazel, who have been a fantastic mentor for me. I am really indebted to her for encouraging my research and for allowing me to grow as a research scientist. Her advice on both research as well as on my career have been invaluable. I could not have imagined having a better advisor and mentor for my master study.

I would like to thank the rest of my thesis committee; Assoc. Prof. Dr. Oguzhan Gunduz and Asst. Prof. Dr. Doruk Dogu for their supports.

I would like to thank Ahmet Ozan Basar, for his advices and supports for my thesis.

Finally, I must express my very profound gratitude to my family, to my friends and to my cat Pomi, for everything that they had done for me. This accomplishment would not have been possible without them.

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LIST OF ABBREVIATIONS

PCL	-	Poly (Ɛ-caprolactone)
TE	-	Tissue Engineering
ES	-	Electrospinning
SC	-	PCL Solvent Cast Scaffold
SC E	-	PCL Electrospun Fiber Coated PCL Solvent Cast
SC EP	-	PCL Electrospun Fiber Patterns on PCL Solvent Cast
ATR–FTIR	-	Attenuated Total Reflection Fourier Transform Infrared
SEM	-	Scanning Electron Microscopy
CA	-	Contact Angle

CHAPTER 1

Introduction

Recently, the guidance of cells by manipulation of cellular movement and adhesion in vitro by topographic alterations and/or biological cues is a promising tool for biological or biomedical studies. It can be done by either exposing the surface to a biological molecules or manipulating the surface geometry. When the cells are seeded onto the geometrically or biologically modified surface the choices made by cells such as morphology chances, alignment or attraction can be monitored [1]. Also, experimental control of increased growth of the cells and their connections to the surfaces is become important for biotechnological applications because of the fact that their cooperation with the host tissue can be restrained by the designated patterns, pathways or networks for the formation of circuits. So that, cell guidance is a useful approach for creating biosensors, prosthesis and implants containing grafted cells, vascular tubes or neural networks [2].

Surface patterning is one of the ways for the production of these tools and it is based on the creating physically and/or chemically distinguishable regions on the substrate surfaces. Pattern creation on surfaces has been used on fabrics and papers since the ancient times, but it owes its modern development to the electronics and semiconductor technology. These advanced techniques such as mask-based photolithography and focused electron beam lithography were adopted in biomedical studies in order to manipulate the surfaces of biomaterials to locate cells accurately. Thus, surface patterning enables the examination of the relationship between cells, biological molecules and the surface material [3, 4]. Besides the surface chemistry or geometry, the structures where the cells will hold, grow and multiply should also be selected according to the intended applications.

Extracellular matrix (ECM) is the natural three dimensional habitat in which the cells live, proliferate and differentiate. Today, as more information about the structure of

the ECM and the functioning of the cells in it is obtained, it has also been possible to create structures similar to ECM in a laboratory environment [5]. Scaffolds are the artificial structures which mimic the natural ECM functions, enable regeneration of the cells and grant structural support to the tissue. In order to be used in biotechnical applications, scaffolds must be non-toxic, biocompatible, porous and biodegradable as well as it should give enough mechanical support to target tissue. A wide range of materials can be used as scaffold base material including metals, ceramics, polymers and composites. However, especially for soft tissue applications polymer scaffolds are superior [6-8]. Polymers are divided into two groups as natural and synthetic polymers according to their sources. However, synthetic polymers are preferred because their properties such as biocompatibility and strength can be tailored in the laboratory environment and they have no risk of disease transmission [9].

Considering the recent studies, poly(ε-caprolactone) (PCL) is one of the most frequently mentioned synthetic polymers for tissue engineering studies because of its low production cost, controllable mechanical properties and degradation rate, low toxicity, exclusive blending capacity with other materials and good permeability to chemicals [10]. PCL scaffolds can be produced using techniques such as electrospinning, solvent casting and 3D printing. Among them, the electrospinning is noticeable because it allows the production of porous fiber structures with micro to nano scaled diameters. In addition to that, the price of electrospinning setup is low and the process control is easy when compared to the other conventional fiber fabrication methods such as extrusion [11].

In this study, our approach was the creating surface patterns by direct writing with a mask/stencil and integrating it with the electrospinning process. Firstly, PCL solvent cast film was produced and it was fixed to the collector of the electrospinning device. Afterwards, the stencil with holes on it was attached to the solvent cast film. So that, while the electrospinning process was continuing, patterns were also created on the surface simultaneously. The morphology and the size of the electrospun fibers were analyzed by SEM and the surface wettability was decided by contact angle measurements. Also the chemical composition of the surface of the produced scaffolds was examined by FTIR analysis and strength of the scaffolds was determined via

mechanical testing. Lastly, the effects of the surface patterns on the cell-material interaction and cell behavior were monitored *in vitro* over a 7-day period using mouse fibroblast cells (L929).

CHAPTER 2

Literature Survey

2.1. Tissue Engineering Scaffolds

2.1.1. Tissue Engineering

Nowadays, organ and tissue damages that triggered by illnesses, old age, injuries or any other type of failures are the one of most significant healthcare issues that cannot be ignored by the researchers. Some therapy methods like surgery, mechanical apparatus, drug treatments, manufactured prostheses and transplantations from human or animal origins have been developed to treat those failures or reduce their effects on the living organisms. However those treatment techniques are not adequate for the regeneration or the repair of damaged organs/tissues for all cases. For instance, as a well-known fact that neuronal tissue cannot self-regenerate because it does not carry stem cells [12]. Even though those old-fashioned methods have saved many patients and improved their life quality, they have non-negligible disadvantages like being too expensive because of the rare resources, having high risk for infection and disease transmission, high sensibility for organ/tissue refusal by immune system, and unpredictable long-term usage by the patients. Besides that if the patient is a child or a teenager those problems become more severe because most of those treatments are stationary and they cannot grow simultaneously with the patient's development [13].

In order to overcome these problems, Tissue Engineering (TE) is evolving as a novel and suitable alternative for previously mentioned old-style techniques for the regeneration and repair of a damaged organ and tissue. In 1993, Langer and Vacanti wrote an article titled "Tissue Engineering" where they described Tissue Engineering as "an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function" [14]. It is a multidisciplinary science area that combines the

powers of material science, molecular biology, medical science, chemistry, as well as engineering for replacing/repairing the damaged organs/tissues. In contrast of old-fashioned ways, Tissue Engineering focuses the regeneration of the failed tissues and tries to restore or enhance damaged tissue function instead of replacing it [15].

Tissue Engineering has three major components which are biomaterials, growth factors and living cells and it aims to improve regeneration or repair the function of the failed organ or tissue with these components in combination or alone [16]. Among these three key elements, biomaterials that are used for manufacturing scaffolds provide a vital role. Scaffolds acts as templates for tissue regeneration and guide the growth of the new tissue. Biomaterials that are produced as scaffolds mimic the structure and function of specific tissue types. They act as extracellular matrices (ECMs) of the targeted tissues and support the cell and tissue recovery [17]. Another important factor for tissue engineering is the cells formed by cultivation of cells obtained from human or animal tissues in the culture medium. Specific tissue cells show different characteristics and examination of those features gives the status of the regeneration and improvement. Also when there is a frailty of functional cells, stem cells and progenitor cells, which can differentiate into a collection of phenotypes, are crucial to compensate [18]. Lastly, signalling molecules that are usually function as growth factors bind to the receptors of target cells and they regulate multitude of cellular activities [19].

The complex and difficult-to-understand structure of the human body has led to the formation of categories in parallel with this in tissue engineering. It could be divided classes according to their target tissues, those are; i) Soft Tissue Engineering ii) Hard Tissue Engineering. Soft Tissue Engineering involves with the vascular, skin, cartilage, tendon, ligament, skeletal muscle, and nerve tissues where as Hard Tissue Engineering focuses on bone tissue [20]. Examples of recent studies in these areas are given below:

Tissue Engineering of Vascular Tissue: Gupta and friends prepared bi-layered small diameter silk scaffolds for blood vessel grafts. The inside of the graft is highly porous and produced by molding and lyophilisation. The outer surface of silk tube was coated with nano fibrous Poly (ε-caprolactone) and silk electrospun layer. They made in vitro

experiments and found that their bi-layered tissue engineered vascular graft showed excellent porosity, resilience and biodegradability. Also, in vivo implantations of the grafts in rat aorta revealed long patency duration and remodelling of the grafts by the infiltration of the host cells and extracellular matrix deposition that supports their medical expediency [21].

Tissue Engineering of Skin Tissue: Fernández-Cervantes and colleagues managed to develop scaffolds from a Cassiopea Andromeda jellyfish. They used decellularization process to decrease original cell population and to produce their scaffolds. Their characterizations showed that their jellyfish based scaffolds mimic human skin tissue and allow optimal adhesion and support the proliferation of dermal fibroblast cells [22].

Tissue Engineering of Cartilage Tissue: Sharifi and friends worked with native cartilage extracellular matrix components Gelatin (GEL-CS) and Chondroitin Sulphate and utilize a co-electrospinning technique with Poly (ε-caprolactone) (PCL) to prepare composite scaffolds to promote human bone stem cell chondrogenesis. Their in vitro experiments showed that with the increasing GEL-CS content the cells on the scaffolds performed better chondrogenesis differentiation and they are the new candidates for further cartilage tissue engineering applications [23].

Tissue Engineering of Tendon Tissue: Lee and colleagues manufactured different ratios of polycaprolactone (PCL)/gelatin (GE) blends and tried to optimize the electrospin process to get highly aligned nano-fibers for tenogenic differentiation. They made in vitro and in vivo experiments and found that the highest cell proliferation rate was belong to PCL/GE (9:1) group. However PCL/GE (7:3) blend showed the highest tenogenic differentiation so that it showed more potential for being used for future tendon tissue engineering applications [24].

Tissue Engineering of Ligament Tissue: Gwiazda et. al. work on the effects of fiber arrangements on melt electrospinning writing technique on ligament tissue. They used different kinds of patterns and monitored the cell orientation in vitro and they found that cells orientate on the aligned pattern. Also they managed to produce large

cellularized structures which consist of bone-ligament-bone (BLB) constructs with complicated shapes by melt electrospinning writing method [25].

Tissue Engineering of Skeletal Muscle Tissue: Sicari et. al. focused the case of volumetric muscle loss (VML) and produced an acellular scaffold that is originated from porcine urinary bladder. They made *in vivo* experiments with mice and human patients who suffer VML and found that their porcine based scaffolds can enhance the formation of new muscular tissue with the utilisation of perivascular stem cells. [26].

Tissue Engineering of Neural Tissue: Jing and coworkers prepared a conductive conduit which contains parallel aligned conductive fibers and they tested it's capacity of improving peripheral nerve regeneration *in vivo.* They chose conductive polypyrrole (PPY) as shell and deposit in onto parallel-aligned electrospun poly (lactide co-glycolide) (PLGA) nano fibers. The aligned fibers revealed an important orientation by guiding the nerve cells. Also, their *in vivo* experiments showed that aligned conductive fibers in a conductive conduit could utilize an optimal micro environment for the growth of neural cells because of their transmittance capacities [27].

Tissue Engineering of Bone Tissue: Neufurth et. al. managed to use 3D printing technique to prepare an active bio ink consisting of micro-particles produced from corporal inorganic polymer polyphosphate (polyP) and Ca^{+2} by successfully combining it with Poly (ε-caprolactone) (PCL). They made experiments with bone like Sa-Os cells in vitro and found that their granular hybrid material is applicable for the production of bioprinted scaffolds which shows not only good mechanical properties but also promising morphogenetic potential [28].

2.1.2. Scaffolds

Over the previous decades, there was a wide range of studies and researches that have been focused on the production of tissue-like and regenerative materials that lead to the generation of scaffolds having similar features of original organs and tissues. Those scaffolds are necessary for the repairment, regeneration or the replacement of the human body tissues that are damaged because of the traumas, diseases and genetic abnormality that damages and degenerates normal functions of them [5]. The term scaffold can be defined as the "temporary home" of the cells that bio mimics the

original tissue functions and extracellular matrix architecture by granting the three dimensional structural support and enabling the regeneration of cells. Also, for enhancing the cell growth and regeneration of natural tissue, scaffolds can discharge some bioactive molecules (growth factors), drugs, enzymes and genes to the specific regions [6, 7]. There are number of features that a good scaffold must carry in order to be used in biomedical applications. These properties can be listed as; i) biocompatibility, for causing minimum toxicity or inflammatory effects to target tissue, ii) having high surface to volume ratio for encouraging cell adhesion, extracellular matrix deposition and cell-material interplay, iii) being permeable and/or porous enough to allow the transportation of nutrients, growth factors, gases that are mandatory for the survival, growth and differentiation of the cell, iv) being biodegradable in a controlled manner depending on the regeneration rate of the target tissue/organ, v) being strong enough to provide the necessary structural integrity while the target tissue or organ is regenerating [8]. Since each patient's and application's demands are various it is challenging to modify all of these described concerns together. When a specific demand is full filled, there is a high chance that another will be affected negatively. For example, despite the fact that the porous structure is favourable for many aspects for regulating the cell-material functions, they cannot be used for load-carrying applications as a result of their poor mechanical features. Therefore, researchers have tried to obtain a synthesis of these mentioned requirements by, i) developing new materials such as bio-ceramics or metal-ceramic and ceramic-polymer composites, ii) proposing new processing techniques or altering the current ones in order to get desired features [29]. The main production methods for preparing scaffolds are solvent casting, particulate leaching, melt molding, freeze drying, electro-deposition, rapid prototyping, gas/batch foaming and phase separation [30]. As a consequence of these production methods, scaffolds could be in many forms such as; porous, hydrogel, micro-spherical, fibrous and acellular [8].

For any tissue type, there are some concerns for determining the applicability or designing the biomaterials that will be used in tissue engineering;

Biocompatibility: It is the major concern for tissue engineering studies that use biomaterials for regenerative purposes since that the designed scaffold will be

implanted into the human body. Accordingly, proper biocompatibility will enhance the attachment of cells by making them function naturally so that the cells migrate to the trauma/injury or implant site to facilitate the proliferation of cells for the new tissue establishment. The most important features observed in biocompatibility are the absence or minimal amount of immune system reaction at the target to which produced scaffold/implant is applied [31].

Biodegradability: The basic working mechanism of tissue engineering is based on the ability of cells in the human body to secrete enough enzymes to dissolve biomaterial and to build their own ECM in damaged areas. Thus, while biomaterials are gradually disappearing, cells also construct matrices of their own unique structure on which they can grow and develop. This digestion of biomaterial occurs in many applications except some metal implants such as joint or hip implants. The macrophages which are elements of the immune system show excellent support for this type of degradation. Also, the waste materials produced by the digestion of biomaterials must not be toxic and harmful to the tissue and organs [5].

Mechanical Properties: Since every tissue or organ has its unique purpose, each of them has mechanical properties that integrate with its own functions. That means that the mechanical features are depending on the anatomy and the physiology of the organ/tissue. Also, while implanting the scaffold to the body by surgery, handling usually becomes problematic. The scaffolds must be strong enough to avoid failures during surgery and while being used by the patient. Since each patient's height, weight, age etc. are different, designing optimal tools depending on those factors are gained importance. For example, the regeneration rate of tissues of the elder people is slower. In addition to that, porosity is usually a wanted property for scaffolds but it lowers the homogenous distribution of the mechanical properties throughout the scaffold [32].

Scaffold Structure: The structure of the scaffold is a significant parameter for the repair of failed tissue or organ. Scaffolds needs to be porous i) to ensure the penetration of cells into the scaffold ii) to enable the necessary nutrient transportation to the cells, ECM and within the scaffold itself iii) to send the waste products away from the scaffold. Also the pore size of the produced scaffold exerts an influence to the cell

attachment and infiltration. Besides that, the morphology and the alignment of the cells could be determined by the scaffold topography [33].

Application Cost: Over the past decades, implants and scaffolds are become popular but the cost is still a significant factor for their production and applications, as these materials are hardly ever produced by mass production [31]. Also, national health systems are not eager to pay serious amounts for them and getting help for payment requires significant amounts of regulations and paperwork. Because of that, scientists are focused to produce the biomedical scaffolds that can be directly offered to market with reasonable costs [34].

2.1.2.1. Scaffolds Based on Their Materials

For the production of tissue engineering scaffolds, selection of proper materials and production methods are main issues. With the emergence of tissue engineering, many materials including metals, polymers, composites and ceramics were studied to manufacture scaffolds [7].

2.1.2.1.1. Metallic Scaffolds

Metals and metal alloys were mainly used in orthopaedics due to their exceptional mechanical features like corrosion and wear resistance, ductility, stiffness, electrical and thermal conductivity Most of the metals are bio-inert which means that they make minimum interaction with the body and cells. Also producing end products from unprocessed materials is easy. Metal-based scaffolding materials such as iron oxide, titanium, silver, gold and magnesium are often used for hard tissue applications in the forms of fibers, rods, shells, wires, spheres and shells [35-37].

In Tissue engineering applications, usage of metals are advantageous because of the ease of taking forms, obtaining requested compositions, having excellent resistance to compression and fatigue [38]. Also surface properties of the metals permit the functionalization by polymers and biomolecules such as antibodies, peptides and nucleic acids [39]. Metallic scaffolds can also be porous and these porous metals can be used for bone regeneration applications. They are favourable bone and dental graft materials due to the fact that they enhance the stability and integrity of the grafts. Metal-based scaffolds are especially desired for their load carrying capacities and

being porous helps reducing the stiffness and stress shielding [40]. Despite the fact that the metals can be considered for the best choice for implants for their advanced load carrying features they have some limitations, i) they are not biodegradable, however it is a desired concern for some applications where restoration of damaged tissue is onerous or not controllable, ii) not enough integrity with biomolecules, iii) some metals and alloys is vulnerable for wear and corrosion, iv) they can release toxic ions, v) the need of surgical operations for implementing and removal of the implants. However, these limitations may vary depending on the character of the application; the desired feature in one application may have negative effects for the other application [38]. There are many processes available in order to produce porous metal-based scaffolds such anodization, thermal decomposition, plasma spraying and sintering [41].

Titanium is one of the most common metallic biomaterials with the perfect biocompatibility and excellent corrosion resistance. Titanium can be alloyed with many alloys such as manganese, aluminium, vanadium, cobalt, molybdenum, niobium, nickel or tantalum to increase its toughness and to change its transition temperature [42]. Table 2.1 shows some of the recently made researches about metallic scaffolds.

Table 2.1 Recent researches involving with metallic scaffolds.

Used Metal or Alloy	**Scaffold Form**	**Target Tissue**	**Findings**	**Reference**
Nickel	Porous	-	Good mechanical properties and reduced stress concentration	[43]
Ti/Al/V Alloy	Porous	-	Decreased colonization of bacteria	[44]

Stainless Steel	Porous	Bone	Enhanced osteogenic gene activity but reduced cell proliferation	[45]

2.1.2.1.2 Ceramic Scaffolds

The first studies about bio-ceramics were done in 1920s and it was involved with the calcium phosphate and its applications with hard tissue. Also, hydroxyapatite which is widely used in hard tissue repair, was started to be mentioned frequently in the 1970s [46]. Bio-ceramic materials can collaborate with the human body by; i) being bio-active, they could be exposed to the chemical reactions or a make a strong connection with the neighbouring tissue, ii) being bio-inert, they do not make any connection or contact with its environment, iii) being bio-resorbable, they could be dissolved by the organism and used for the supplement of needed constituents for the regeneration or repair of the tissue [47]. For example, when transferred to the damaged bone sections, calcium phosphate (CaP) nanoparticles can enhance the hard tissue restoration and they can be the nano-scaled carriers of growth factors which are responsible for bone formation. Besides, the ceramic-based scaffolds have desirable features such as being bioactive, stiff (by having high elastic modulus), biodegradable and biocompatible. However most of them are brittle; thus, they face with forming and shaping limitations [48]. Ceramic materials that used in biomedical applications can be classified in three groups, i) bio-inert ceramics such as aluminium oxide and zirconium oxide, ii) bio-active ceramics such as sintered hydroxyapatite (at high temperatures), iii) bio-resorbable ceramics such as tricalcium phosphate and (sintered hydroxyapatite at low temperatures). The bio-resorbable ceramics are mainly preferred for bone graft applications and for porous scaffolds. For example, since natural bone contains large amounts of hydroxyapatite it is suitable for bone tissue engineering because it can promote the original chemistry and mineral structure of the bone [49]. Generally, CaPs and bio-active glasses are utilized for restoration of hard tissues as a result of their excellent bone formation performance. These bio-ceramics can be manufactured by

different methods such as wet chemistry, biological deposition, sol-gel and crystallization from an aqueous solution [50].

The bio-ceramics give poor load bearing properties when compared to the metals because of their inelastic features and for having incompatible Young's modulus and compression strength with the natural bone. Also, polymeric scaffolds could be biodegradable and biocompatible as well as bio-ceramics, but they lack the necessary mechanical strength [51]. Table 2.2 shows some of the recently made researches about ceramic scaffolds.

Table 2.2 Recent researches involving with ceramic scaffolds.

Used Ceramic(s)	Scaffold Form	Target Tissue	Findings	Reference
Titanium oxide	Porous	Bone	Enhanced biocompatibility and cell proliferation	[52]
Hydroxyapatite/β-tricalcium phosphate	Porous	Endometrial	Increased cell proliferation and not causing embryo-toxicity	[53]
Silicocarnotite/α-tricalciumphosphate	Porous	Bone	Similarity with natural bone structure, promoting the bone growth	[54]

2.1.2.1.3. Polymeric Scaffolds

Polymers can be briefly defined as the chemical compounds that are long chained or large molecules that are composed from the linkage of repeating small units called

monomers. Numerous monomers are covalently bonded to each other to form the polymer. Most of the polymers are organic compounds mainly consist of carbon, oxygen, hydrogen and nitrogen atoms. They take an important place for tissue engineering studies for variety of tissue types due to the fact that the polymer based scaffolds enhances cell attachment, adhesion, growth and differentiation and yields with the new tissue generation in three dimensions. The common structural concerns of scaffolds such as high surface to volume ratio, porosity, pore shape, pore size are important key parameters for cell migration, movement, adhesion, and formation of new and healthy tissues. The polymers can be divided into two categories on the basis of their origins; i) natural polymers, ii) synthetic polymers [9, 55].

2.1.2.1.3.1. Natural Scaffolding Polymers

Natural polymers are a subclass of polymers that can be created by the living plants, animals or micro-organisms [56]. They are one of the vital parts of our daily lives; because of the fact that the human body is based on natural polymers such as nucleic acids and proteins. Natural polymers can be divided into two main categories; i) polysaccharides, ii) polypeptides. The polysaccharides such as agarose, alginate, chitosan and hyaluronic acid contain sugar monomer chains that are connected by glycosidic bonding, whilst polypeptides such as collagen, gelatin and silk contain amino acid chains that are connected by peptide bonding. The plant or animal originated polypeptides exhibit better scaffolding capabilities when compared to the polysaccharides [12, 57].

First implementations of natural polymers were in the pharmaceutical and cosmetic industry applications as well as being used as medicine for regenerative purposes. Natural polymers possess some unique properties for TE applications such as having high stability, controllable and variable solubility, low risk of immunogenicity, exclusive bio- and cyto-compatibility, antigenicity and being specific to targeted tissue [58]. When compared to the synthetic and semisynthetic polymers, natural polymers promote better bio-mimicing of the extracellular matrix and tissue interaction due to their selfsameness of the natural tissues [30]. Although the aforementioned benefits and wide range of applications, natural polymers exhibit some negative features; i) since they are nature originated, they are vulnerable for contamination by microbes,

ii) it is hard to control the hydration rate, iii) their production parameters varies with the applications due to the fact that they have complicated structures and shapes, iv) their production is expensive, especially for hyaluronic acid and collagen, v) their resources are limited [59]. Processing natural polypeptide polymers such as chitosan and cellulose could be troublesome because they suffer from poor mechanical properties. In addition to that, due to their low stability, the rate of degradation and catabolization is much higher when compared to the surrounding tissue. For the purpose of overcoming these obstacles, scientists seek for new horizons by modifying the functionality and structure of the natural polymers in order to get convenient degradation rates and good structural, chemical and mechanical features that are manageable for wide range of biomedical applications. Those so-called manipulation methods could be; i) crosslinking, ii) physical alteration, iii) taking advantage of synthetic and semi synthetic polymers for producing natural/synthetic or natural/semi-synthetic polymer blends, iv) chemical alteration [12]. Table 2.3 shows some of the recently made researches about natural polymer-based scaffolds.

Table 2.3 Recent researches involving with natural polymers.

Used natural polymer(s)	**Scaffold Form**	**Target Tissue**	**Findings**	**Reference**
Sodium alginate	Hydrogel	Skin	Good drug delivery agent, promoting would healing	[60]
Chitosan and Silk proteins	Porous Hydrogel	Muscle	Nontoxic scaffolds enhanced cell attachment and growth	[61]
Collagen/gelatine/chitosan	Porous	-	Exclusive anti-microbial and anti-oxidant features	[62]

2.1.2.1.3.2. Synthetic Scaffolding Polymers

Synthetic polymers are commonly manufactured in large amounts with controllable functions and molecular weights, yet they are relatively cheap when compared to the natural polymers. Nonetheless, the major concerns about the synthetic polymers in tissue engineering applications are the deficiency of biocompatibility such as absence of biological signals as well as being biodegradable in most of the situations. Synthetic polymers exhibit better mechanical properties than nature-based polymers, such as having high tensile and compression strength and elasticity. Also these mechanical properties can be easy modified by using different approaches such as changing solution and process parameters [12]. The brief comparison of natural and synthetic polymers is given in Table 2.4.

Table 2.4 A brief comparison of synthetic and natural polymers.

Synthetic Polymers	Natural Polymers
Man-made	Found in nature
Used significantly for almost 130 years	Used by even the early humans
Akin, but non-identical repetition	Monomers repeat identically
Properties can be controlled	Properties cannot be controlled because of natural reactions
Some of them are biodegradable	Generally biodegradable
Lack of biocompatibility	Biocompatible
Safe for microbes contamination	Vulnerable for microbes contamination
The polymer chain backbone can be carbon, oxygen or nitrogen	The polymer chain backbone is mostly carbon
Problematic eco-friendliness	Eco-friendly

Source: [9]

The synthetic polymers can be divided into five major groups; i) polycarbonates, ii) polyamides, iii) polyesters, iv) polyurethanes, v) phenol-Formaldehyde based polymers [63]. However the most used synthetic polymers belongs to the polyesters and they are; poly(ε-caprolactone) (PCL), poly(lactic acid) (PLA), poly(glycolic acid)

(PGA), and poly(dimethylsiloxane) (PDMS). These biomaterials are approved by U.S. Food and Drug Administration (FDA) and they are exclusively practiced for drug delivery and scaffolding applications. Those polymers are advantageous for following reasons; i) they can be degraded via hydrolysis or by cell enzymes such as esterase, ii) the chemical, compositional and structural features of them can be modified for specific applications. Even with these benefits, the major obstacle is biocompatibility. For example, the absence of necessary biomolecules yields with poor cell-material interactions and reduces cell attachment. In addition to that, synthetic polymers can be transformed to some undesired and harmful compounds like acids. If these transformations are not controlled, the acidity of the surrounding media increases and causes unwanted encapsulation of fibers and inflammations throughout the tissue. Besides that, in many studies some harmful organic solvents are used for preparing initial solutions of synthetic polymers, the residues of these solvents may cause toxic effects to cells *in vitro* and to host tissue *in vivo*. In order to overcome biocompatibility limitations following methods are commonly used; i) chemical alterations and ii) surface modifications, ii) biological alterations, iv) making polymer blends v) mineralization [12, 57]. Table 2.5 shows some of the recently made researches about synthetic polymer-based scaffolds.

Table 2.5 Recent researches involving with synthetic polymers.

Used synthetic polymers	Scaffold Form	Target Tissue(s)	Findings	Reference
PLA and γ-PGA	Fibrous	Skin	The core-shell fibers increase wound healing	[64]
Polyvinylidene fluoride (PVDF)/ PCL	Porous	Neural	Promotes nerve regeneration	[65]
Polyethylene glycol (PEG)/ poly(L-lactide) (PLLA)	Fibrous	Bone, Skin	Increased cell attachment and promotion of ECM-like cellular structures	[66]

2.1.2.1.4. Composite Scaffolds

Composite materials are made of at least two separate materials such as ceramic/polymer and polymer/metal. In the previous subsections, the disadvantages of metallic, ceramic and polymer scaffolding materials are explained. Based on these, it can be said that a single type material cannot be sufficient for TE applications, depending on the area in which it will be used. As a result, composite scaffolds are developed to overcome the problematic sides of those materials. For example, as mentioned, bio-ceramics are very brittle and stiff materials with exclusive biodegradability and biocompatibility; and polymers have nice elasticity and poor mechanical strength. In order to improve processability and elasticity of the ceramics, polymers can be added. Also, metals can be added to give the produced scaffold a load-carrying ability. Therefore, producing composite scaffolds is necessary for achieving optimal mechanical, biological, chemical and structural features to the biomedical scaffolds [8, 67]. Table 2.6 shows some of the recently made researches about synthetic composite scaffolds.

Table 2.6 Recent researches involving with composite materials.

Used materials	**Scaffold Form**	**Target Tissue(s)**	**Findings**	**Reference**
PCL/PLGA/stainless steel	Porous	Infected joint	Antibacterial scaffolds with good drug delivery properties	[68]
Agarose/chitosan/graphene oxide	Porous	Bone	Promoting cell attachment and proliferation	[69]
Sodium Alginate /PEG/ Satureja Cuneifolia	Porous	Skin	Antibacterial scaffolds promote cell growth	[70]

2.2. Surface Patterning

Surface patterning is the usage of surface modification methods in order to get physically and chemically distinguishable regions on the substrate surfaces. The patterning of materials is not a recent case; methods like batik, lithography, block printing and screen printing were used to print patterns on textiles and paper at pre-industrial era. However, the beginning of modern ways of surface patterning methods emerged with the development of electronic field in order to create patterns on the surfaces of semiconductor materials. These techniques were started to be used in the production of biomedical materials in the following years. The ability to accurately define the locations of biomolecules and cells is become possible with the surface patterning techniques. There are a number of criteria that these techniques must meet in order to be used for biomedical purposes. Firstly, these conditions will be explained and then these patterning techniques will be briefly mentioned [3].

2.2.1. Common Concerns of Surface Patterning in Biomedical Applications

Surface patterning is a powerful method that allows the examination of the relationship between cells, biological molecules and the surface material. There are several constrains that guide how the patterning practices should be carried out: [4]

Resolution: It is the shortest feature size that can be produced with a specified production technique. It is also used to know the feature size ranges that the particular method can produce.

Throughput: It refers to the total area that can be produced in a given time for a particular method.

Contrast: It is the measure of which parts of pattern is definitive when compared to the substrate material background. Contrast is usually obtained by sending one material over to another material for patterning.

Bioactivity: When working with biological molecules it is significant to protect the bioactivity. Since the most of the patterning techniques were adapted from electronic field, the use of these techniques on biomolecules and materials can lead to degradation or denaturation because of high temperatures and vacuum.

Durability: When choosing the correct technique for patterning applications another key factor is the consideration of shell life of produced pattern. In addition, the patterned material produced must remain as designed while being used by the end user within the end use conditions. This is particularly important for surfaces that are patterned to provide cell growth on implants.

2.2.2. Patterning Methods

As mentioned before patterning methods that used for biomedical applications were derived from electronics industry. These methods can be examined under three main headings: i) direct-write patterning methods; these are generally establish high resolution with the risk of losing throughput and processing speed. ii) Patterning by mask or masters; unlike direct-write patterning methods, those processes give great processing speed and throughput with the expense of resolution. iii) Self-assembly patterning; it is strong tool for creating extensive surface areas with great resolution. iv) Patterning of three dimensions; it is used to get ECM-like structures and to control the cell environment [4].

2.2.2.1. Direct-write Patterning Methods

These methods fabricate patterns by continuously scanned element throughout the entire surface of the material. Direct-write patterning allows fabricating pattern elements with high resolution and accuracy. The disadvantages of direct-writing are that the processes take too much time to complete and not convenient for patterning large surfaces because of the obligation of writing of the pattern continuously. Those drawbacks can be addressed to the specially designed pens. The usage of different types of pens led to the emergence of different direct writing techniques patterns [71].

2.2.2.1.1. Stylus Writing

It involves of the usage of a rigid stylus for writing the pattern. The examples of stylus writing that are utilized by using atomic force microscopy (AFM) instruments are nano-shaving (nano-engraving), nanoimprinting and dip-pen nanolithography (DPN). These applications are usually expensive since the usage of other tools that are already needed for other things such as AFM is needed [72].

Dip-Pen Nanolithography: This method is based on the simple idea of using quills for writing. The atomic force microscopy tips can be filled with desired solutions and the material surface is scanned with the AFM probe in order to write the pattern. The wide range of biomolecules such as viruses, enzymes, biopolymers and proteins can be used the form patterns on the substrate materials [72].

Engraving: The imprinting or engraving the surface to form patterns could be achieved by using hard and rigid stylus such as AFM tip to scratch or mark the desired geometries. Since the patterning mentality of engraving is very similar to direct writing it has same concerns and advantages [72].

Inkjet Printing: It refers to any technique that based on delivering fluid through a small tip to form the desired pattern. The small droplets that coming from nozzle can be forced to form a pattern by electrical forces or mechanically or thermally activated pulses. Biological inks that are solutions of biomolecules or chemicals can be used with the inkjet printing. The high throughput on large surfaces, contact free patterning, high spatial accuracy and availability of using multiple nozzle heads simultaneously are the main advantages of inkjet printing. [73]

2.2.2.1.2. Patterning with Beams

An energetic beam is send to the surface in order to create patterns in these techniques. The limiting factor is the spot size of the beam which determines the spatial resolution. Those energy sources could be light for direct write photolithography, electrons for beam lithography or ions for focused ion beam lithography [4].

Direct Write Photolithography: The most common energy source that could be used for patterning is light. Focused photon beams are send to the surface for direct write photolithography meaning that the optical diffraction limit dictates the spot size. Direct light photolithography is favorable method for biomolecules since it can be used at aqueous conditions. The patterns are created by either physical changes or photochemical mechanisms of the surface material. Photochemical reactions occur because of the fact that the photons can stimulate the photoactive molecules locally to directly form patterns or other elements to create patterns [71]. Also, a laser can be modified to form patterns by physical reactions on the surface of the substrate material.

This technique is similar to AFM but unlike AFM, polymers and resins can be used. Polymer and resin materials may become molten or deformed due to the high energy beam [74].

Focused Electron Beam Lithography: It has the similar principles with direct writing lithography with light. Nonetheless, instead of focused photons focused electron beam lithography (EBL) uses electron beams used as the tool for creating patterns on to the materials with e-beam sensitivity. The electron beams can trigger functionalization or crosslinking of defined surface regions. Since the used beams in EBL have shorter wavelengths than that is used for direct light photolithography it has shown higher resolution. So that it is a strong technique that can produce small feature sizes in nanoscale (10-100 nm). One of the main concerns of EBL is that the scanning electron microscopes can be modified for EBL but they are expensive. Also, since EBL is not a routine method, configuration of SEM to EBL and reconfiguration of EBL to SEM might be a forced labor. In addition, the patterning via EBL is very slow and it can only write patterns to small parts of the surface that means EBL has low throughput. For this reason, it is a perfect tool for forming high resolution patterns for small areas. Besides that, since EBL is basically a modified SEM application it needs high vacuum to proceed which restrains the usage of the biomolecules [75].

Focused Ion Beam Lithography: Another way of direct writing patterning is emerged from using focused ion beam (FIB). It is very much alike with electron beam lithography and has similar benefits and limitations. One of those limitations is that in focused ion beam lithography, heavy ions like gallium ions are send to the surface instead of electrons in electron beam lithography. It is a harmful technique since the bombardment of ions yields with atomic sputtering from the surface. So that it is used as a milling method in order to engrave substance surface [4].

2.2.2.2. Patterning via Masks

Since it is the core mentality of microelectronic field, patterning with the help of the masks is highly established area. They are also used for patterning of biological molecules and viable cells. Briefly, a mask is a geometrically designed apparatus which allows to traverse the radiation and fields or it can be defined as a template that can physically protects some defined areas of the underlying material from etching

agents or inks. The most used form of the mask patterning is the masking of light and it is called as photolithography although photolithography can be done without masks with direct writing techniques as mentioned previously. Apart from light, masking can be done with radiation, bio inks, chemicals or biological etchers. It is an impressive and functional tool for producing geometrically designed surfaces because of; i) high throughput, large regions can be patterned. ii) Layers can be obtained by ensuing masking steps with different deposition or etching stage. iii) Complex patterns can be produced because the only limiting factor is the dimensions predicted by the mask [75].

Mask-based Photolithography: The mask used for photolithographic applications must have opaque and transparent regions. Those masks can be metal, glass or flexible materials like transparencies. The most vital requirement of the masks which will be used in photolithography is that it needs to allow light to pass through the specially designated areas and so the pattern that is dictated by the mask is obtained under the mask. However, the term photolithography is often used to describe a set of processes that are used for semiconductor industry, not just forming patterns by mask and light. A silicon wafer that carries the spin coated polymer based photoresist is utilized for mask based photolithography. Photoresist become active with the exposure to light and creates patterns in a development solution on to an underlying substrate material. Photoresists are divided into two groups: i) Positive photoresist, the specific regions of it dissolves in the development solution when it is exposed to light but the regions that do not see light, maintain their integrity without dissolution. ii) Negative photoresist, utilized with the opposing mentality; the photoresist regions that are exposed to light show decreased solubility in the development solution and the not exposed to light areas will dissolve and uncover the underlying material with the patterned structure. Finally, after the removal of the unwanted parts, the emergence of an etching step in order to dissolve the thin metal oxide films or deposition stage that allows to preferentially coating of the material occurs [4].

2.2.2.3. Master-based Patterning

In batch processes, a template is used for the replication of patterns is called a master. Stiff masters can be directly used for printing patterns on to substrate surfaces. But,

the most common usage of masters is for forming a mold from an elastomeric material to use it for molding the desired patterns. Polymeric materials are often cured for obtaining the molds with the presence of master which transfers its geometry to the newly manufactured mold. Molds can create patterns by printing features on surfaces by using inks that can be biomolecular. First ink is applied to the mold and then the ink makes contact to the surface of the material for the purpose of transferring the pattern of the mold. Master-based patterning is a cheap and simple process with capability of producing large areas with high speed but it has lower resolution when compared to the direct writing methods. Another way to use masters for patterning is similar to stylus writing methods. Unlike the stylus writing which can write only one pattern at a time, masters allow the imprinting of many shapes at once. In order to obtain the desired geometry by deformation, substrate material needs to be soft such as polymers and mold material must be hard [4].

2.2.2.4. Patterning by Polymeric Self-Assembly

Self-assembly of colloids and polymers is powerful and effortless technique if initiated properly. The patterns are created by experiments that benefit from intermolecular forces that obligate the polymer or colloids to separate or agglomerate in a geometrically specified pattern compelled by the minimum free energy of the entire system. Self-assembly of polymers can be used for large surfaces and yields with high resolution. There are three main types of self-assembly of polymers; nanosphere lithography, block copolymer and magnetic self-assembly [76].

Nanosphere Lithography: It is the nano-scale self-assembly of particles in order to create template or pattern. A solution that contains dispersed nanospheres that comprise of a polymer is poured into a flat surface. When specific conditions are met, a monolayer that contains hexagonal close-packed nanospheres is formed by self-assembly with solvent evaporation and cause the void formation between nanospheres. That monolayer contains nanospheres and it can be used as mask in order to apply the pattern to the underlying substrate [77].

Self-assembly of Block Co-polymer: It is the self-assembly of molecules of copolymers. The patterning is done with a block copolymer that has more than one blocks that are not miscible to each other. The solvent is put onto the surface and while

the solvent that contains block copolymers evaporates, the micro segregation processes are takes place. This micro segregation then creates the high resolution patterns that can coat the large surfaces but the defects are usually occur [3].

Self-assembly by Magnetic Forces: When magnetic field is applied to the small particles having magnetic features, those small particles can be oriented or aligned. Magnetic particles can be directly sent to surface to form the pattern or they can be attached to a part of a cell to make cell move to the designed region [78].

2.2.2.5. 3D Patterning

It is a relatively new patterning technique and the biggest motivation of applying three dimensional patterning is the fact that the cells exits in a 3D environment called ECM. Since the cell related culture studies are usually done at flat surfaces like flasks where they can make interaction with the neighboring cells only. Since the planar surfaces are not represent the 3D cell environment properties, the findings of cell culture studies often become irrelevant. 3D patterning can be used for obtaining ECM like microenvironments as well as designing the biosensors for diagnostics and makes microfluidic devices more controllable. The most of the previously explained processing methods can be used for patterning the surfaces of 3D structure, but there is some advances techniques that needs to be mentioned [4].

***Multiple-Photon Lithography (MLP*)**:** It is a direct write technique that uses lasers in order to obtain well defined shapes and patterns. The photosensitive or photo-initiator materials are excited by multiple-photons for the purpose of photo-crosslinking or photo-polymerization of the materials by the highly instantaneous focused pulsed laser photon beams. The laser focus starts the photochemical actions in small volumes called voxel. The photosensitive materials are cured locally in the solution of photoactive materials as so the voxel becomes three dimensional. The surrounding uncured solution is removed to obtain well defined and rigid 3D shapes. Since working with large areas requires higher power, it is a costly method for patterning large areas [4].

Holograph Lithography: It is a 3D patterning technique that projects the 3D images with the help of intersecting patterns of focused laser beams. The complex shapes can be created by the manipulation of following properties of intersecting lasers: number

of separate laser beams, their alignment with each other, polarization, amplitude and phase. Even if the patterning is done very quick when compared to previously explained techniques that requires mask or direct writing, the beam alignment process is complex and optical components and devices are expensive [4, 79].

Table 2.7 shows the recent studies involving with the various patterning techniques for various purposes.

Table 2.7 Recent studies related with biomaterial surface patterning.

Material	Patterning Method	Findings	Reference
Au/Ti, Peptides	Dip Pen Lithography	Peptide patterns helps Controlling Cell Behavior	[80]
Poly(ethylene glycol) diacrylate (PEGDA)	Direct Write Photolit.	Enhanced colonization of neural cells	[81]
Titanium dioxide, Glass	Electron Beam Lit.	Cells tend to attach and proliferate onto the TiO_2 patterns not glass	[82]
Silicon based electrode	Focused Ion Beam Lithography	Nano-structures of Si can be obtained by FIB and they are candidates for neural tissue regen. applications	[83]
Polydimethylsiloxane (PDMS) and FITC proteins	Holographic Lithography	3D polymeric and protein microstructures are obtained simultaneously	[79]
PCL/F127	Inkjet Printing	The cells printed onto the scaffold accurately and started to proliferate	[84]

PDMS	Mask-based Photolit.	Aligned pillars with uniform surface were successfully produced, they are promising candidates for cell studies	[85]
Gelatin methacrylate (GelMA)	Master-based Patterning	Produced patterns and and GelMA provide biological signals for the treatment of corneal endothelial dysfunctions	[86]
SU-8 photoresist	Nanosphere Lithography	Pattern on the SU-8 was promoted the neuronal cell growth	[87]
Amphiphilic block copolymer (BCP) and BCP/magnetic particles (MNPs)	Self-assembly by Magnetic Forces	Magnetic particles were speed up the self-assembly process and produced micro tubes found to be candidates for cell studies	[78]

2.3. Cell Guidance

Extracellular matrix (ECM) is the natural network in which cells live through and producing special structures similar to ECM is one of the main targets of tissue engineering studies. The idea of cell guidance comes into prominence as more information becomes available on the biological control of ECM microenvironment and cellular function. The guidance of cellular attachment, network formation and migration by surface cues *in vitro* become significant for biotechnical applications and researches [1]. The guidance of cells could be done by several methods;

Biological Signals: Surface cues are created to stimulate cells on the substrate surface with ECM biological molecules such as growth factors and proteins. Thus, the desired pathways to guide cells are used with the help of biological molecules and chemicals. For example, cells could be seeded on surfaces shaped by patterns consisting of a

combination of different matrix proteins. Thus, morphological changes, orientations and interaction of cells with each other and effects of different matrix proteins on cellular behavior can be observed [2].

Topographic Alterations: The capability of guiding cells and their development with the surface related cues are crucial for multicellular fitting and design. Another way to achieve this is to create geometrical shape and texture alterations on the surface. For instance, multicellular assembly of vascular tubes consists of neuronal networks or endoletal cells that concatenate each other throughout the defined patterns of the substrate surface. The aforementioned network structure can be accomplished by a surface that contains both antiadhesive and adhesive areas. The cells forced to attach and proliferate on the permitted adhesive sections while avoiding the surface with antiadhesive properties [2].

Microfluidic Devices: The *in vitro* control of cellular attachment, connection and growth can be managed by grafted cells that are present in the artificial implants or prosthesis. The connection between grafted cells and its surrounding tissue could be achieved by providing predetermined pathways that form circuits. Cell guidance with the help of microfluidic devices is a promising way to create biosensors because single cells could be directed to the defined paths on the surface [2].

However, it should not be overlooked that surface chemistry and surface geometry are not completely different topics. Biochemical manipulations on the surface have effects on the physical features like roughness, topography, and elastic properties of the surface. Besides that, topographical differences may interact with the differential deposition of culture medium contents and/or cell originated matrix proteins and causing their surface to acquire biochemical properties [2].

2.3.1. Cell Guidance Mechanisms

In order to understand cell guidance phenomena it is better to understand cell-substrate interactions, cell structures and mechanisms need to be mentioned.

2.3.1.1. The Cell Cytoskeleton

The internal order, morphology and movability of cells are identified by two main structural factors; firstly, cytoskeleton: the framework within the cell and secondly, the connection tools of cells that they can attach to their surrounding area.

The cell cytoskeleton composes of three protein originated systems: microtubules, microfilaments and intermediate microfilaments. The chain reaction of decomposition and construction of the cytoskeleton parts and the cell adhesion permit the cell to change its morphology, to react its microenvironment, to locate, to split, to conduct position of its components, and to arrange information transmission between the cell organelles [2]. The main features of those three mentioned protein based structures:

Microtubules: These are consist of wide range of α-β heterodimer tubulin proteins. It is believed that they are responsible for the organization of intermediate thread framework [88].

Microfilaments: They are composed of miyosin, actin and several related proteins. They crosslink the proteins in order to form comprehensive gel-like structures. Their shape does not change permanently unless a long-term mechanical force is applied.

Intermediate Microfilaments: They are divided into four major groups on the basis of their definitive proteins. *Type 1* consist of keratins, Type *2* are vimentins (for osteoblast and fibroblast like cells), *Type 3* are neuro-filament complexes and *Type 4* consist of nuclear laminins.

Some recent studies on cell guidance are briefly discussed below:

- Fraioli and coworkers studied with chemically tailored surfaces that are patterned by integrin binding peptides and found that those biochemicals stimulates osteogenic differentiation *in vitro* and bone formation *in vivo* with the help of stem cells [89].
- Nuhn et al. worked with the radially aligned and randomly created collagen fibers. Mesenchymal cells were responded to the aligned fibers and started to migrate but amoeboid cells did not migrate directionally. They also found that

the increased stiffness by glycation paralyzed the directionality of mesenchymal cells [90].

- Xia and colleagues focused to astrocyte cells that are considered important for neuroregeneration. They designed aligned and non-aligned electrospun poly(methyl methacrylate) scaffolds and seeded the astrocyte cells *in vitro*. They found that aligned PMMA nanofibers supports the cell adherence and cell growth of astrocytes [91].
- Du et al. aimed for neuronal repair and regeneration. They produced aligned fibers of PCL (polycaprolactone) and found that those fibers exhibit intense guidance signals. Also they found that the neurite elongation and adjustment favored when electrical field applied [92].
- Zhang and coworkers produced 3D photocatalytic PCL surfaces by melt electrowriting method and altered the spacings between the PCL fibers as well as decreasing the texture angles from 90º to 30º. Additionally, they managed to achieve optoelectronic modification and neural stimulation by the addition of graphene oxide and graphitic carbon nitride which is a photo catalyst of visible-light. Those alterations make them able to produce nerve guidance conduits (NGCs). They managed to direct neurites along the micro pattern and the anisotropic geometry of those conduits were addressed for peripheral nerve regeneration [93].
- Buskermolen and friends found that the contact guidance of cells happen because cells tend to avoid gaps between designed grooves and so that they move along the direction of groove lines [94].

2.3.1.2. Cell Attachment to Surface

In cell culture studies, cells adhere to the surfaces on which they are seeded as well as attaching to each the other. The closest cell-substratum interaction is around 10-20 nanometers and it is named as focal adhesions. The connection mechanisms involves with many protein complexes that have sequenced arrays dictated within ECM. The transmembrane glycoproteins such as integrin connect to the ECM components like laminin or fibronectin [2].

2.3.1.3. Cell Motility and Development of Focal Adhesions

The cellular movement is the result of the relations between cell cytoskeleton and attachment surface. For example, actin filaments can be torn off by cytochalasins. Manipulating cell with cytochalasins inhibits the cell's cytokinesis, motion, phagocytosis, and generation of micro spikes. On the other hand, for securing the actin filaments phalloidin can be utilized and it stops the cell migration. Those experiments highlights that the cell movement and shape changes are actin based chain of events [95].

When the cell movement mechanism is examined in detail, the following data are obtained in the literature. Even before cell movement begins, the cell has many thin extensions (lamellopodia) that bulge in every direction. These protrusions contain actin-based short filaments called micro spikes. If two lamellopodia that are in opposition to each other provide a strong interaction, their synergy yields cell to be bipolar. The overgrowth of lamellopodia is restrained by the other actin extensions that are located along the cell membrane. Therefore, these contentious forces cause the cell to take a more stretch form. Afterwards, the real cell locomotion is propagated because of cell movement. While the back end of the cell is stationary, lamelopodium, which contains micro spikes in the excited front, expands. New series of adhesions are formed at the leading edge of the cell. Finally, the cell begins to move on in the direction of the newly generated adhesions [96].

2.4. Electrospinning

Electrospinning (ES) or electrostatic spinning technique is a versatile method for producing nanofibers by the high electrical voltage [97]. It is considered as a variation of electrospraying and nano to micro sized fibers can be easily fabricated via ES. Although both electrospinning and electrospraying need high voltage for liquid jet to occur; in electrospinning method, continuous fibers are generated by evaporation of the solvent, while small sized droplets are obtained because of the low viscosity of the solution in the electrospraying process [98].

The first steps of electrospinnning depends on the interest of liquids to electrostatical forces, which can be considered the basis of ES, were carried out in the 16th century

[99]. Almost four centuries later, the first trials of electrostatic spinning complated succesfully with the fabrication of fibers [100]. However towards the end of the 20th century, ES technique has gained its popularity and many studies conducted by this method because it was discovered that this technique can produce nanofibers from organic polymer solutions. Nonetheless, in contrast to the initial ES applications; nanoscaled fibers with controlled interporosity, diameter and order are fabricated via the modern ways of electrospinning. Also, and the price of set up is low and the process control is easy when compared to the other conventional fiber fabrication methods such as extrusion [11, 101].

The ES process depends on the overcoming the surface tension of the droplets of by the applied electrical force [102]. The circular droplet at the tip of the electrospinning needle is forced to take a small conical from (known as Taylor cone) by the high voltage [103]. When the applied voltage becomes dominant to the surfce tension of the liquid droplet, the solution starts the come from the formed Taylor cone as a jet. If the initial is consist of solution, with the initial release as jet, the solvent starts to evaporate. It is important to use ventitalion at the chamber for proper evaporation of the solvent, because some of the solvents could be harmful for human health [97, 104].

2.4.1. Typical Electrospinning Device Setups

A typical electrospinning device consists of; i) syringe (or spinneret) with small diametered metal tip where polymer melt/solution is come throught, ii) pumping device for constant flow rate, iii) high voltage source for creating electrical field, iv) collector where fibers deposit. The collector can be rotating or fixed depending on the application [11, 97]. The most common ES device set up are horizontal or vertical depending on where syringe and the collector is located. But for some applications complex fibrous structures are needed so that different ES device setups are developed. For example, for enhanced and aligned cell attachment and growth, the nanofibres can be aligned by using rotating collector or two parallel collectors [105, 106].

2.4.2. Factors Affecting the ES Process

There are several vital parameters that affects the many features of the fabricated product, such as; topography, mechanical properties and fiber diameter. Those factors

can be divided as i) ES solution parameters, ii) process parameters, iii) environmental conditions. The solution parameters include role of solvent, surface tension of the solution, molecular weight, viscosity, conductivity and concentration whereas important processing conditions are applied voltage, feed rate and distance between collector and syringe metal tip. The environmental factors are the temperature and humidy of the area where ES process takes place [107, 108].

2.4.2.1. ES Solution Parameters

Role of Solvent: The morphology of nanofibers is affected by the type of solvent, because solvent changes the electrical and physical properties of the solution. The type of solvent is also impotant for obtaining beadless products [108].

Surface Tension of the Solution: It is important for the generation of smooth fiber structures. The low surface tension of the solution allows obtaining fibers at relatively low voltages whereas the high surface tension may disturb the stability of the jet and yields with the beads [109, 110].

Conductivity: Commonly, the polymer solutions are conductive because of their ion content. Conductivity of the solution is based on several factors such as; i) type of the solvent and polymer ii) type of the present ions. When the voltage applied, the solutions with high conductivity exposed to higher tensile forces and yields with decreased fiber diameters. On the other hand solutions with low conductivity results the formation of beads and in inadequate elongation of fibers [97, 111].

The synergy of Molecular Weight, Viscosity and Polymer Concentration: Depending on the application, the MW of the polymer should be suitable for the application and electrospinning process. Because at low MWs, polymer solutions cannot form continuos fibers and beads occur whereas, very high MWs inhibit formation of the jet because of the high viscosity. Also, the viscosity of the prepared solution is mostly depends on the MW of the polymer. The usage of polymers with low MW yields with low solution viscosity and vice versa. The viscosity of the solution must be optimised too, because if viscosity is too high or low it can be problematic. For instance, low viscosity yields with merged fibers and this can be solved by addition of polymer. In addition to that, the concentration of the solution determines the spinnability of the

prepared solution. The concentration of the solution must be optimezed too; if the polymer content is low (dilute solution) instead of the fibers beads are occured and if the polymer content is high (concentrated solution) the e-spinning would not start because of the high viscosity. The lenght and diameter of the fibers are increases with the increasing concentration, if the concentration of the solution is in the optimal concentration range [97, 110, 112].

2.4.2.2. Process Parameters

Distance between Collector and Syringe Metal Tip: The collector and needle tip must be at optimum distance for each polymer type. For example if the distance is too short, there may not be enough time for the evaporation of the solvent and beads may occur [113].

Supplied Voltage: It is the most important factor for ES process, since it determines the strenght of electrical forces that are required for the formation of fibers by overcoming the surface tension of the solution. Different types of polymers show different behaviour to voltage changes, and there is thus the optimization of supplied voltage is vital. For some polymers such as PVA, high voltage erlarges the fiber diameter while for several polymers such as cellulose, high voltage decreases fiber diameter [114, 115].

Flow/Feed Rate: The optimization of flow rate is crucial because the stable Taylor cone must be maintained throughout the process for the fibers with uniform properties. Nonetleless, the low flow rate gives more time for solvent evaporation in order to avoid formation of beads. In addition to that, when flow rate increase the pore size of the fibers increase depending on that [97, 116].

2.4.2.3. Environmental Factors

Humidity: The humidity affects the evaporation rate of the solvent and porosity of the fibers. If humidity increases the porosity of the fibers also increase. Also, it helps the ejection of the solution from the tip of the needle because at low humidity levels may cause the solution in the needle tip to dry and clog the tip [117].

Temperature: The changes in temperature affect the viscosity of the solution and evaporation rate of the solvent. Since the kinetical energy of particles increases with increasing temperature and viscosity decreases, as a result it causes the stretching of the fibers [97].

2.5. Poly(ε-caprolactone) (PCL)

PCL or poly(ε-caprolactone) is one of the polyesters with biodegradability and the first synthesis of it dates back to 1930s, with the emergence of synthesis of polyesters [118]. PCL has gained importance during 1980s, with the discovery of the ability of microorganisms to decompose synthetic polymers [119]. It can be produced mainly by two routes; ring opening polymerization reaction of ε-caprolactone, ii) free radical ring opening reactions of 2-methylene-1-3-dioxepane [120]. PCL is a saturated aliphatic polyester with hydrophobic properties, which is composed of hexanoate repeating units. Also, it is a semi-crystalline polymer with thermoplastic nature and exhibits up to 70% crystallinity depending on its molecular weight (MW). Its melting point (T_m) varies between 59°C to 64°C and its glass transition temperature (T_g) is -60°C. Because of these, PCL occurs in rubbery form at room temperatures yielding high toughness, and exclusive mechanical properties depending of its MW. Its average molecular weight can be tailored within the range of 3,000 to 80,000 grams/mole depending on the application. It does not cause toxicity to tissues and hosts, which means it is suitable for biomedical applications. Thus, It has wide-range of application history and it is used for; i) drug delivery purposes, ii) production of resorbable surgical sutures, iii) for scaffolds for tissue engineering [121]. However, its biggest problem is that it lacks of required bio-functionality and load-bearing properties for some biomedical applications, but these can be solved by blending it with different materials [122]. The degradation of PCL can be done by hydrolysis of its ester bonds or by microorganisms and this degradation process takes 2 to 3 years [121, 123]. It has the slowest degradation period amongst all of the polyesters, because PCL contains hydrophobic $–CH_2$ branches in its monomers [124]. It has good solubility in solvents such as chloroform, benzene, dichloromethane and its insoluble in methanol [125]. PCL is used for many biomedical applications as a result of its low production cost, controllable mechanical properties and degradation rate, exclusive blending capacity with other materials and good permeability to chemicals. As a result of its low T_m,

good thermal stability, low cost and appropriate viscoelasticity; it is suitable for techniques involving with melting. In addition to that, since it is soluble in wide range of non-polar solvents, it can be used for techniques that are specialised for using organic solutions [10]. The main scaffolding methods can be listed as, i) solvent casting, ii) solvent casting/particulate leaching, iii) electrospinning, iv) 3D printing, v) phase separation, iv) melt electrospinning [121]. Besides that, PCL is used in many biomedical fields including bone, tendon, cartilage, ligament, blood vessel, skin and nerve engineering [126]. Depending on previously mentioned production techniques, PCL can be in the form of film, fibers, microspheres, hydrogel and nanoparticles. For example, PCL fibers are commonly produced by electrospinning technique for medical (i.e. scaffolds for tissues) or drug delivery applications [127]. Also, PCL films that are produced by solvent casting are preferred in scaffolds, because they have large surface areas and slow degradation rate. Films are especially demanded in drug delivery applications because the film layer withstands the motions of segments and changes in crystallinity [128].

CHAPTER 3

Experimental Study

3.1. Materials

PCL (Poly (Ɛ-caprolactone) (MW=80,000 g/mol, linear) was purchased from Sigma-Aldrich. Dichloromethane was used as solvent for solvent casting of PCL, where chloroform and methanol were used for electrospinning of PCL. Glass petri dishes that used for solvent casting were obtained from Interlab (Ankara, Turkey). The stencils are polyester fabric with rounded holes on it. For cell culture studies, ATCC CCL-1 L929 mouse fibroblast cells were kindly taken from Department of Bioengineering, Kirikkale University (Kirikkale, Turkey). DMEM (Dulbecco's Modified Eagle Medium), FBS (fetal bovine serum), L-glutamine, penicillin/streptomycin, BSA (bovine serum albumin) and Phosphate buffer saline (PBS) tablets were bought from Amresco (Solon, USA). Ethanol (99% purity, vol./vol.), (3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide) MTT powder, Trypsin/EDTA solution at 0.25% (w./v.), Triton X-100, glutaraldehyde, dimethylsulfoxide (DMSO), hexamethyldisilazane (HMDS) (99% purity, vol./vol.), hydrochloric acid (HCl) were bought from Sigma-Aldrich (St. Louis, USA). Furthermore, the stains Acridine Orange dye (AO) and Propidium Iodide dye (PI) were bought from Sigma-Aldrich (St. Louis, USA).

3.2. Preparation of the Samples

3.2.1 Production of Solvent Cast PCL

The 2D PCL scaffolds were prepared by solvent casting procedure. PCL (7.5 w/w%) was dissolved in dichloromethane by magnetically stirring for 2 h at room temperature. The solutions in 10 ml volume were poured into 9 cm petri dishes and placed on a flat surface. Aluminum foil with the needle holes in it covered onto the petri dish for slow evaporation and kept for 2 days at room temperature. At the end of 2 days the samples

are put into 40°C heated oven to dry for 17 h. Finally, the dried PCL solvent cast samples are kept in room temperature for additional 3 days in order to get rid of residual solvents. After this period, the samples were stored in desiccator until the further usage.

3.2.2. Preparation of the Electrospinning Solutions

Solution parameters for electrospinning of PCL were adopted from previous works in our laboratory. Initially, PCL pellets were weighted (15% (wt./vol.)) carefully and dissolved in a chloroform and methanol mixture with the 3:1 volume ratio at 30°C. The solution was mixed by magnetic stirrer for 3 hours until clear homogenized solution was observed. Finally, the PCL solution was kept in room temperature for 10 minutes to eliminate air bubbles present in the solution.

3.2.2.1. Electrospinning Optimizations for Patterning

In order to obtain electrospinning of PCL onto solvent cast PCL, the electrospinning parameters were optimized. Those parameters are applied voltage, flow rate and the distance between collector-needle. For the case of electrospinning of PCL onto solvent cast without stencil, the parameters that were used were shown in Table 3.1 Besides, the electrospinning parameters with the stencil were shown in Table 3.2. For all of the cases, a single jet electrospinning setup was used and many experiments to determine the electrospinning process parameters were done. Also, the used stencil with the 5 mm diametered holes is shown at Figure 3.1.

Figure 3.1 The mask/stencil used to create patterns

Table 3.1 Electrospinning process parameters of PCL onto solvent cast without stencil.

Sample	Experiment Number	Collector to Needle Distance (cm)	Flow Rate (µL/min)	Voltage (kV)	Result
PCL onto Solvent Cast (SC E)	**1**	8	10	10	No Spinning
	2	8	10	15	No Spinning
	3	8	13	10	No Spinning
	4	10	13	15	No Spinning
	5	10	15	20	No Spinning
	6	10	10	25	No Spinning
	7	12	12	20	No Spinning
	8	12	15	25	High Voltage
	9	12	15	20	No Spinning
	10	15	18	26	High voltage
	11	15	10	10	No Spinning
	12	15	10	20	No Spinning
	13	17	10	15	No Spinning
	14	*17*	*12*	*18*	*Spinned*
	15	17	15	22	High Voltage
	16	20	12	15	No Spinning
	17	20	15	20	No Spinning
	18	20	15	25	High Voltage

Table 3.2 Electrospinning parameters of PCL onto solvent cast with polyester stencil.

Sample	Experiment Number	Collector to Needle Distance (cm)	Flow Rate (µL/min)	Voltage (kV)	Result
PCL through plastic stencil (SC EP)	**1**	6	10	5	No Spinning
	2	6	12	8	No Spinning
	3	6	12	10	No Spinning
	4	8	10	6	No Spinning
	5	8	12	12	No Spinning
	6	8	15	10	No Spinning
	7	10	10	10	No Spinning
	8	10	12	15	No Spinning
	9	10	15	10	No Spinning
	10	10	10	15	No Spinning
	11	12	10	10	No Spinning
	12	12	13	22	No Spinning
	13	12	10	12	No Spinning
	14	15	10	15	No Spinning
	15	15	15	25	High Voltage
	16	18	12	15	No Spinning
	17	18	15	20	No Spinning
	18	20	10	15	No Spinning
	19	*20*	*12*	*20*	*Spinned*

	20	20	15	25	High Voltage

3.3. Characterizations of the PCL Scaffolds

3.3.1. Thickness Measurements

The thickness of prepared solvent cast, patterned and non-patterned electrospun scaffolds were measured using caliper (0.25 mm, outside micrometer, USA). For patterned scaffolds the measurements were repeated for circular electrospun patterns and non-patterned solvent cast surface. Thicknesses of each unique sample were measured 5 times and those values were used to obtain average thinknesses the different patterned samples.

3.3.2. Scanning Electron Microscopy (SEM) Imaging

The patterns (electropun parts) and solvent cast surfaces' detailed morphological properties such as average fiber diameter, porosity and average pore size were examined by Scanning Electron Microscope (SEM), S-4100 Hitachi (Tokyo, Japan). The electron microscope was set with the 10 mm working distance and of 5 kV accelerating voltage. The scanning electron microscope micrographs were used for the determination of the average fiber diameter, porosity and the average pore size with the help of ImageJ software. For each of the different patterns, 20 measurements are done in 5 repeats.

3.3.3. Contact Angle Measurements

The wettability of the produced materials was determined using CA goniometry Phoenix 300 apparatus (Suwon, South Korea). The CA values are measured from i) Elecrospun parts ii) Solvent cast parts. All experiments were done with ultra-pure water. The contact angle values were obtained as a function of surface tension by the measurement of the surface tension of the water droplets that were put on the specific locations of the samples.

3.3.4 Fourier-transform Infrared Spectroscopy (FTIR)

The surface composition was analyzed with Fourier-transform Infrared Spectroscopy (FTIR) technique. The device used for measurements was Bruker IFS 66/S

HYPERION 1000 (Germany). The scan resolution of 4 cm^{-1} at the range from 4000 cm^{-1} to 500 cm^{-1} was used for getting the characteristic PCL peaks.

3.3.5. Mechanical Properties

An universal testing apparatus (Zwick/Roell Z250) (Germany) was used to measure mechanical properties of the prepared three different samples. The universal testing apparatus was set to 100 N and 0.1 N pre-load was applied. Also the cross-head speed was utilized to 5 mm/min and the gauge length was set to 20 mm. All of the fabricated samples were cut into dog-bone shape. The dimension of the dog bone was 30 mm height and 4 mm width. From the results of mechanical tests, tensile strength, elastic modulus and percent strain at break values were obtained. Also, elastastic modulus of the scaffolds were determined by regression technique between 0.5% - 1% elongation values.

3.4. Cell Culture Assays

Cell-material interactions, the difference between solvent cast and elecrospun PCL parts and the cell guidance throughout the patterns were examined with the help of mouse fibroblast cells line (L929 ATCC CCL-1). The medium for all cell culture tests were same and consist of DMEM, FBS (10% v/v), penicillin+streptomycin solution (1% v/v) and L-glutamine (1%v/v). The scaffolds were cut into circular shapes with 1 cm diameters. Then newly-cut materials were sterilized with UV for half an hour. For better cell to sample adhesion, walls of the well plates (tissue culture polystyrene-TCPS) were covered with parafilm. The samples were put into the well plates and the mouse fibroblast cells were seeded onto the prepared scaffolds with the initial concentration of 5x 10^5 cells/ml. The cell-seeded scaffolds were kept in incubator which was set to 37°C and 5% CO_2 for a week. Also, TCPS was used as control group for all material types and assays. The following assays were done: cell attachment test, MTT assay for cell viability, fluorescence imaging and SEM microscopy imaging.

3.4.1 Cell Attachment Assay

Cell Attachment assay was done by haemocytometric counting method for 180 minutes by 30 minutes intervals: 30 m, 60 m, 120 m, 150 m and 180 m. Firstly, after described intervals were passed the unattached cells were taken away by removing the

medium from the each well. Secondly, the sample materials were kept in incubator (37°C and 5% CO_2) for 5 minutes with Trypsin/EDTA solution (0.25% wt/vol) and attached cells were removed. Finally, the leftover cells were counted by trypan blue dye which gives blue color with the viable cells, for each of the 30 minutes intervals. The results were given as the ratio of the viable cells to initially seeded cells.

3.4.2 MTT Assay

The cell viability of fibroblast (L929) cells on prepared scaffolds were determined quantitively by colorimetric MTT (3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide) assay starting with the end of the first day of initial seeding and followed by the end of the third, fifth and seventh days. Firstly, the 24 well plates were coated with parafilm and the samples were put into the wells and determined number of cells was seeded onto the samples. The TCPS was used for control group for solvent cast (SC), electrospun PCL coated solvent cast (SC E) and patterned electrospun coated solvent cast samples (SC EP). After the incubation period (37°C, 5% CO_2), all the media inside the wells were discarded and scaffolds were rinsed with PBS solution three times. 600 µl fresh medium and 60 µL MTT solution (5 mg/ml in PBS solution) were added onto newly washed samples and kept in an incubator (37°C, 5% CO_2) for 3 h. After the incubation period, purple media which contain formazan crystals were obtained because of the reduction of MTT by living cells. The medium on the scaffolds was discarded and the samples were taken to clean wells and 200 µl DMSO was put onto the samples in order to dissolve formed formazan crystals and kept in the incubator for additional 1 h. Finally, the media from the wells were taken and the absorbance values of the solution were measured at 540 nm by Dynamica LEDETECT96 microplate reader. All of the measurements were done in triplicates.

3.4.3 Fluorescence Microscopy Analysis

Fluorescence Microscopy Analysis was done to get the information about cell attachment and cell proliferation on/into prepared scaffolds. Firstly, for the staining, AO (25 µl/ml in ethanol) and PI (25 µl/ml in ethanol) solutions were prepared. After that, dedicated scaffolds for the third and seventh days of the cell culture were taken and all the media inside the wells were discarded and the scaffolds were carefully washed with PBS solution three times. The fixation of the cells on/inside the materials

was done with 4 % paraformaldehyde for 30 minutes and stained with AO/PI 1:1 (v./v.) solution and kept in the dark room for 10 min. Fluorescence images were immediately taken by the images Fluorescent Microscope (AMG EVOS-FL, USA).

3.4.4 SEM Imaging

SEM images were taken on the 3rd and 7th days of the cell culture period. Briefly, pre-decided scaffolds were taken from the incubator and all the media was discarded and materials were washed with PBS solution. After that, fixation was handled by a 4% paraformaldehyde solution for 30 minutes. The scaffolds were lost their water content by immersion into RO water/ethanol solutions with the increasing amounts of ethanol up to 100% (v./v.) for 2 mins. Lastly, HDMS was added onto the scaffolds, and they were left to air dry. Then dried scaffolds were taken to Middle East Technical University (METU) - Central Lab. (Turkey) for SEM analysis. Scaffolds were coated with Au-Pt in 3 nm dept since the samples were nonconductive. After coating with a conductive material, the scaffolds were examined by SEM (QUANTA 400F Field Emission SEM).

CHAPTER 4

Results and Discussion

4.1. Production of Scaffolds

In order to produce solvent cast, electrospun coated solvent cast and electrospun patterned solvent cast scaffolds the parameters of both electrospinning and solvent casting were determined from experimental optimizations and literature. These optimized parameters are mentioned in Section 3.2.1 and 3.2.2. The resulting scaffolds are shown in the Figure 4.1. The average diameter of the circular electrospun patterns were calculated as 4.4±0.6 mm and the area fraction of the circular patterns were found as 41.23% via ImageJ software.

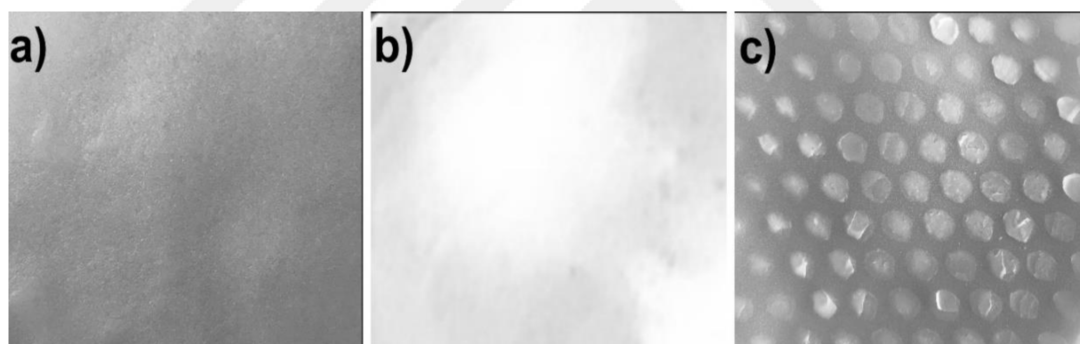

Figure 4.1 a) PCL Solvent Cast Film (SC) b) PCL Electrospun Fiber Coated PCL Solvent Cast Film (SC E) c) PCL Electrospun Fiber Patterns of PCL Solvent Cast Film (SC EP).

4.2. Characterizations of Fabricated Scaffolds

4.2.1. Thickness Measurements

The thicknesses of produced PCL solvent cast films (SC), PCL electrospun fiber coated PCL solvent cast films (SC E) and PCL electrospun fiber patterns on PCL solvent films (SC EP) were measured with laboratory scaled caliper. For SC EP scaffolds thickness values were measured from both circular patterns and non-

patterned surfaces. The thickness values for all fabricated scaffolds lies between 0.16 to 0.28 mm as shown in the Table 4.1 The average thickness of the circular patterns of SC EP was lower than the SC E, because while electrospinning some of the fibers were deposited onto the stencil instead of the underlying PCL solvent cast film.

Table 4.1 Thickness values of prepared scaffolds.

Sample / Measurement	SC	SC E	SC EP	
			Pattern	Non-Patterned
Thickness (mm)	0.162	0.240	0.202	0.174
	0.178	0.271	0.216	0.172
	0.175	0.230	0.213	0.167
	0.167	0.246	0.198	0.177
	0.166	0.280	0.231	0.163
Average Thickness (µm)	169.6±5	249.4±18	212±11	170.6±5

4.2.2. Average Fiber Diameter, Average Inter-Fiber Pore Size and Porosity

The electrospinnability of the PCL onto solvent cast film is affected by the flow rate, needle to collector distance, the solution concentration and applied voltage as indicated before. These parameters also affect the diameter of electrospun fibers and the pore size. The SEM images are processed with ImageJ software (USA) in order to measure the average fiber diameter. For the average fiber diameter, pore size and porosity measurements the solvent cast part was assumed as non-porous surface and its morphology can be seen at Figure 4.1. The average fiber diameter, pore size and porosity measurement results are shown in Table 4.2. The average fiber diameters of SC E and SC EP were found 182±51 nm and 186±50 nm, respectively. Looking at the previous works of our lab and literature, it is seen that the average diameter of the electrospun PCL fibers is approximately twice the values found [129]. This could be because of the change in the applied voltage and/or using non-conductive material at the collector [97, 130]. However, the porosity of the produced electrospun fibers was found 39% for SC E and 45% for SC EP. Also the average pore sizes of the electrospun

sections of produced scaffolds were measured as 1.310 µm and 1.298 µm for SC E and SC EP respectively. In addition to the SEM images of SC E and SC EP electrospun fibers can be seen at Figure 4.2.

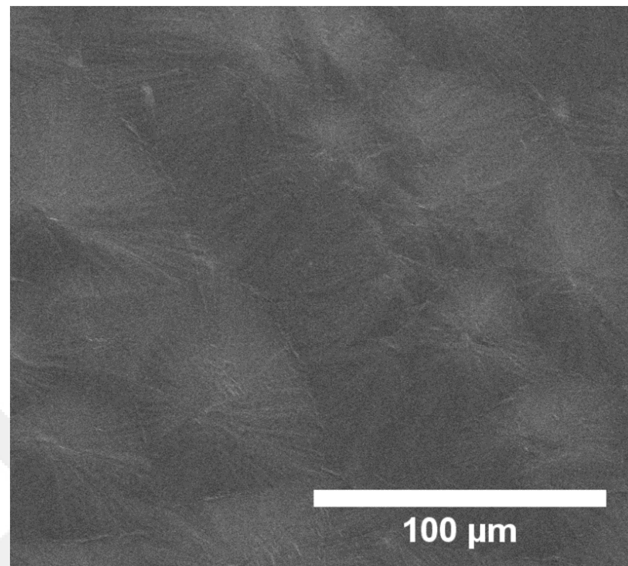

Figure 4.2 SEM image of PCL SC scaffold.

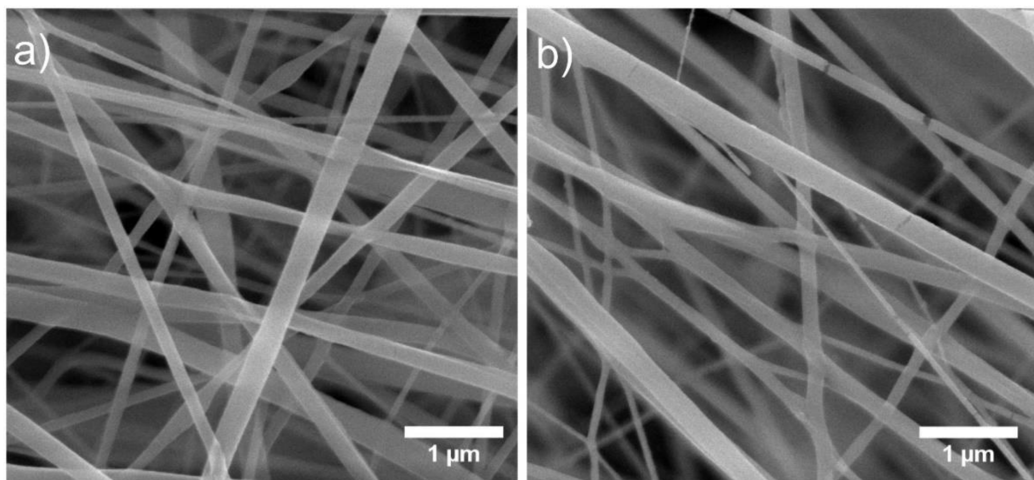

Figure 4.3 SEM images of a) SC E electrospun fibers b) SC EP electrospun fibers.

Table 4.2 Average pore size, porosity and fiber diameters of electrospun fibers.

Sample	Porosity (%)	Average Pore Size (μm)	Average Fiber Diameter (nm)
SC E	39	1.310	182±51
SC EP	45	1.298	186±50

4.2.3. Determination of Surface Wettability

In order to express the surface wettability of produced scaffolds CA (°) values were measured and the results are shown at Table 4.3. The solvent cast scaffold surface had a moderate CA value of ~59°. On the other hand the electrospun parts of the SC E and SC EP had the CA values of ~110° and ~85° respectively. Also contact angle values of solvent cast parts of SC EP were measured and found similar to SC. It can be said from the results, that the electrospun parts were more hydrophobic than the solvent cast parts. The difference between the CA (°) values of SC EP and SC E may have resulted because of the thin layer of fibers in SC EP and more porous structure of it when compared to the SC E. Therefore, contact angle values of SC EP fibers were measured between SC and SC E contact angle values.

Table 4.3 Contact angle values of scaffolds.

Sample		Contact Angle (°)
SC		59.36 ± 1.4
SC E		110.79 ± 5.2
SC EP	Pattern	85.60 ± 3.2
	Non-Patterned	60.17 ± 0.9

4.2.4. Fourier-Transform Infrared Spectroscopy (FTIR) Analyses

The chemical composition and the bonds of a molecule of the material can be determined by Fourier-transform Infrared Spectroscopy (FTIR). The molecular formula of the pure PCL is $(C_6H_{10}O_2)_n$ and this structure contains bonds between

carbon and hydrogen and double bonds between carbon and oxygen which affects the characteristic peaks of the material [131]. The FTIR spectra of produced scaffolds are shown at Figure 4.4. The FTIR spectrum of non-blended PCL shows the characteristic doublet peaks between 2800 to 3000 cm^{-1} wavelengths because of the C—H bonds stretching of the methylne groups. At that region, 2945 cm^{-1} wavelength corresponds the asymmetric stretching of the >CH_2 and the 2865 cm^{-1} wavelength shows the symmetric stretching of the >CH_2 group. There is a singlet peak between 1720-1730 cm^{-1} wavelength which is the characteristic peak of the carbonyl group and the produced scaffolds gives this peak at 1721 cm^{-1} wavelength which is caused by the stretching of the >C=O group. In addition to that, >CH_2 bending can be observed at 1470 cm^{-1} and the >CH_2 wagging at 1365 cm^{-1} in Figure 4.4. Besides that, the symmetric and asymmetric stretching of the C—O—C can be seen at 1163 cm^{-1} and 1238 cm^{-1} wavelengths, respectively [132, 133].

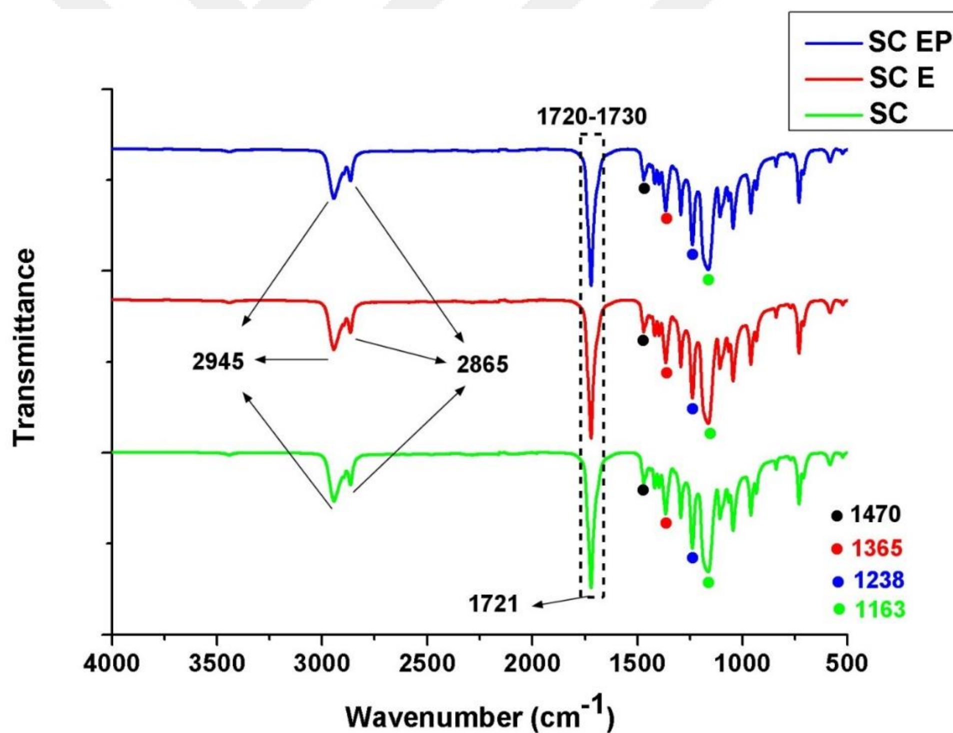

Figure 4.4 FTIR Spectra of produced PCL scaffolds (green line corresponds the SC, red line corresponds the SC E and blue line corresponds the SC EP scaffold).

4.2.5. Mechanical Properties

The mechanical properties such as elastic modulus, ultimate tensile strength and elongation at break of the scaffolds are important parameters for biomedical applications [134]. The mechanical properties of manufactured scaffolds can be seen at Table 4.4. The solvent cast film shows brittle behavior and since it is the dominant layer for SC E and SC EP, those scaffolds also gives low elongation at break (%) values. Due to the fact that the both solvent cast and electrospun layers undergoes the tensile strength which is proportional to their volume fractions, the results of the mechanical test show that the SC E scaffolds have the greatest ultimate tensile strength (UTS) value when compared to the others [135].

Table 4.4 Mechanical testing results of SC, SC E and SC EP scaffolds.

Sample	Strain at Break (%)	Tensile Strength (MPa)	Elastic Modulus (MPa)
SC	6.5 ± 0.2	9.12 ± 0.03	226 ± 6.42
SC E	7.71 ± 0.08	11.65 ± 0.12	147.6 ± 4.98
SC EP	6.46 ± 0.4	9.5 ± 0.03	214 + 1.63

4.3. Cell Culture Studies

4.3.1. Cell Attachment Assay

The cell attachment assay is considered as a first step of the cell culture studies and is crucial for the determination of the cell-material interactions and cell behaviors such as proliferation, attachment, cell guidance and differentiation [136]. The percentage of the attached cells on the surfaces of the produced scaffolds after 3 h is shown at Figure 4.5. The level of significance was calculated by Tukey's Test. The level of significance was taken $p < 0.05$, and data were labelled with (*) for $p < 0.05$, (**) for $p < 0.01$, (***) for $p < 0.001$. From the Tukey's Test, all the data were significantly different with each other with $p < 0.001$. According to Figure 4.5, L929 mouse fibroblast cells managed to attach to the surfaces of all three scaffolds and TCPS which was used as control group. Although, very thin electrospun fibers available at the surfaces of SC E and SC EP, they were highly hydrophobic. In literature, it is reported that the high

hydrophobicity causes poor cell attachment to the surface [137]. Thus, SC surfaces which exhibit moderate hydrophilicity has the highest cell attachment ratio when compared to the SC E and SC EP scaffolds. Also, the percentage of the attached cells on TCPS control group was lower than all of the produced scaffolds. Despite the high hydrophobicity of the electrospun layers of SC and SC EP, the porosity of the materials (39% for SC E and 45% for SC EP) is another important factor for cell attachment. The highly porous structure of the produced electrospun layers supports the cell attachment to the surface, as it allows more locations for cells to attach and proliferate [138].

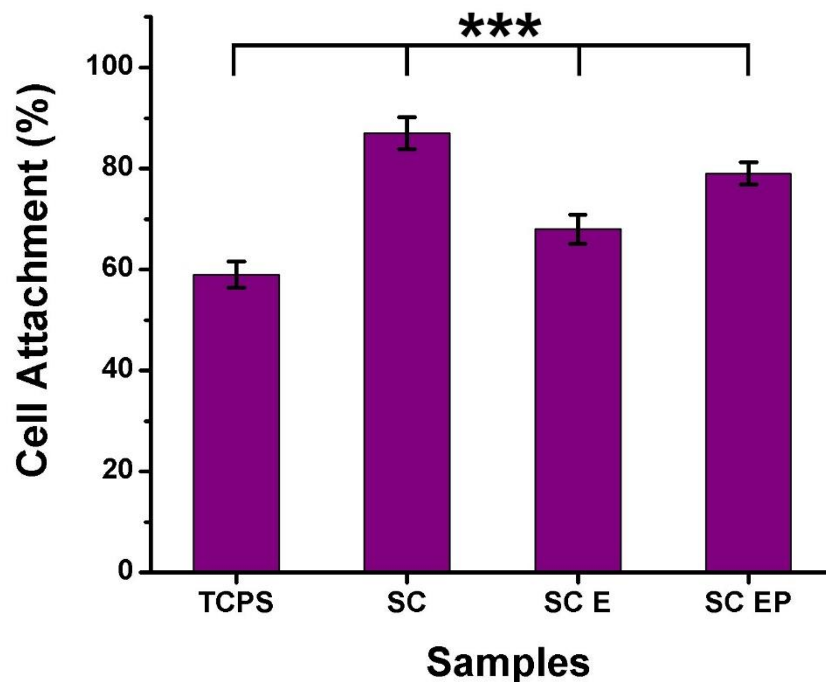

Figure 4.5 The cell attachment percentages of TCPS (control group), SC, SC E and SC EP after 3 h. (***) for $p < 0.001$.

4.3.2. MTT Assay

The cytotoxicity, biocompatibility and cell viability of the prepared scaffolds examined by MTT assay for 7 days period, starting with the 1st day after seeding and at the ends of 3rd , 5th and 7th days. TCPS was used as a control group to get the clue about the performance of the produced scaffolds on the proliferation of the mouse

fibroblast cells. Figure 4.6 shows the MTT assay results. It could be seen that the cells successfully attached to the surfaces of all materials and TCPS at the first day and cells began to proliferate and reached to the maximum amount at the end of cell culture period (7th day). The absorbance values of TCPS control group were lower than the absorbances of the samples for all days. Since fibroblast cells prefer smooth surfaces over rough surfaces [139], they attached to the SC surfaces more at the first day. Also SC E surfaces with electrospun fibers on the surface give the lowest absorbance values at the first day and this means that the fibroblast cells showed less willingness to attach to the electrospun surfaces when compared to the smooth SC surfaces. In addition to that at 1st, 3rd and 7th days of the cell culture, since the SC EP scaffold has both smooth solvent cast and rough electrospun regions its absorbance values lies between the absorbance values of SC and SC E. Also at the early days (1st and 3rd days) this preference of choosing smooth surfaces over rough surfaces of the fibroblast cells could be observed. For 1st, 3rd and 7th days all of the absorbance values were statistically different from each other with $p < 0.001$ (***). On the 5th day, there was a significant difference between control groups and samples, however there was not a statistically significant difference of absorbance values between the samples. This could be happened because of the proliferation of the cells. When the amount of the cells were increased, since the SC E and SC EP had 3D electrospun parts and they were highly porous (39% and 45% respectively) cells might be started to proliferate in/on the 3D electrospun regions more. As mentioned before, having more porous structure yields with more available sites for cell growth and proliferation [138], so that SC E scaffold had the highest and SC had the lowest absorbance values at the last day of the cell culture. Also, since the SC EP had the area fraction of the circular patterns as 41.23%, its absorbance value stands between SC and SC EP for 1st, 3rd and 7th days. On 3rd day, solvent cast smooth surfaces promotes cell attachment and proliferation more than electrospun surfaces, so that SC EP absorbance value was affected positively by solvent cast parts and negatively by electrospun regions. However, on the 7th day, electrospun surfaces encourages cells proliferation by providing more sites because of its porosity, thus it can be said that the electropun patterns were affected the absorbance value positively while solvent cast parts performed poorly when compared to the e-spun regions.

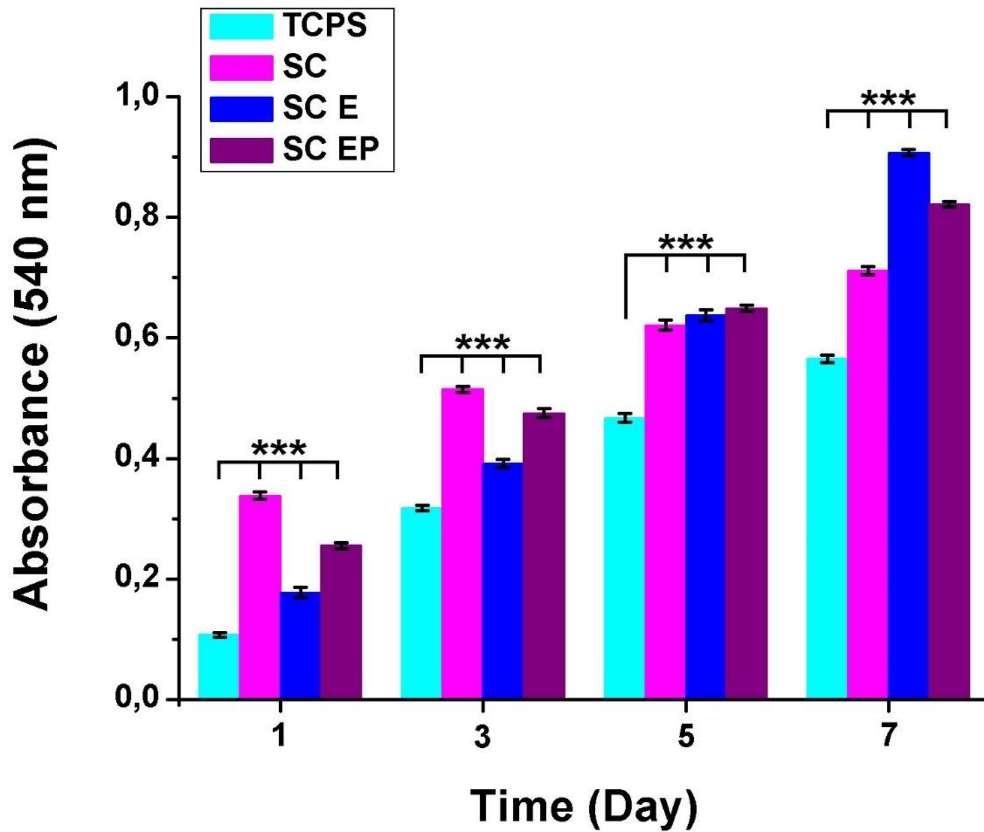

Figure 4.6 Absorbance results of MTT Assay for 1st, 3rd, 5th, 7th days of the cell culture. (***) for $p < 0.001$.

4.3.3. Florescence Microscopy Analyses

Cell proliferation and attachment at the 3rd and 7th days of cell culture are shown at Figure 4.7. The cells on the scaffolds were stained by AO and PI (1:1 v/v) dyes. It could be seen from Figure 4.7 the seeded fibroblast cells had the typical fibroblast morphology during the cell culture period. The viable cells give green color and the cells with poor membrane integrity gives red color at the fluorescence micrographs. Also, it can be observed from Figure 4.7, while the cell density was low for each sample on the 3rd day, the cell density was increased on the 7th day. The florescence imaging of SC EP sample was done from electrospun patterns and solvent cast parts separately because the average pattern diameter (~4.4 mm) was big and it was not possible to capture images showing entire electrospun patterns. Also, it could be seen that the fluorescence images are coherent with the MTT assay findings. For example, viable cell number on the SC E on the 3rd day of cell culture was lowest when compared

to the other samples, but on the last day of cell culture it had the highest viable cells. In addition to that, cell proliferation and attachment of the cells on SC EP patterns looks similar to the SC E micrograph and smooth solvent cast parts of SC EP looks similar to SC scaffold. Thus, it can be said that by introducing patterns to the scaffolds, cell density at the different regions could be manipulated.

4.3.4. Scanning Electron Microscopy (SEM) Imaging

The attachment and morphology of the cells on produced scaffold surfaces were examined with SEM. Figure 4.8 shows the shapes of the mouse fibroblast cells on the different samples. For SC EP, the SEM images were taken from solvent cast and electrospun regions separately. SEM images were also coherent with florescence and MTT analyses, showing that the electrospun surfaces had more cells when compared to the SC parts at the end of the cell culture period.

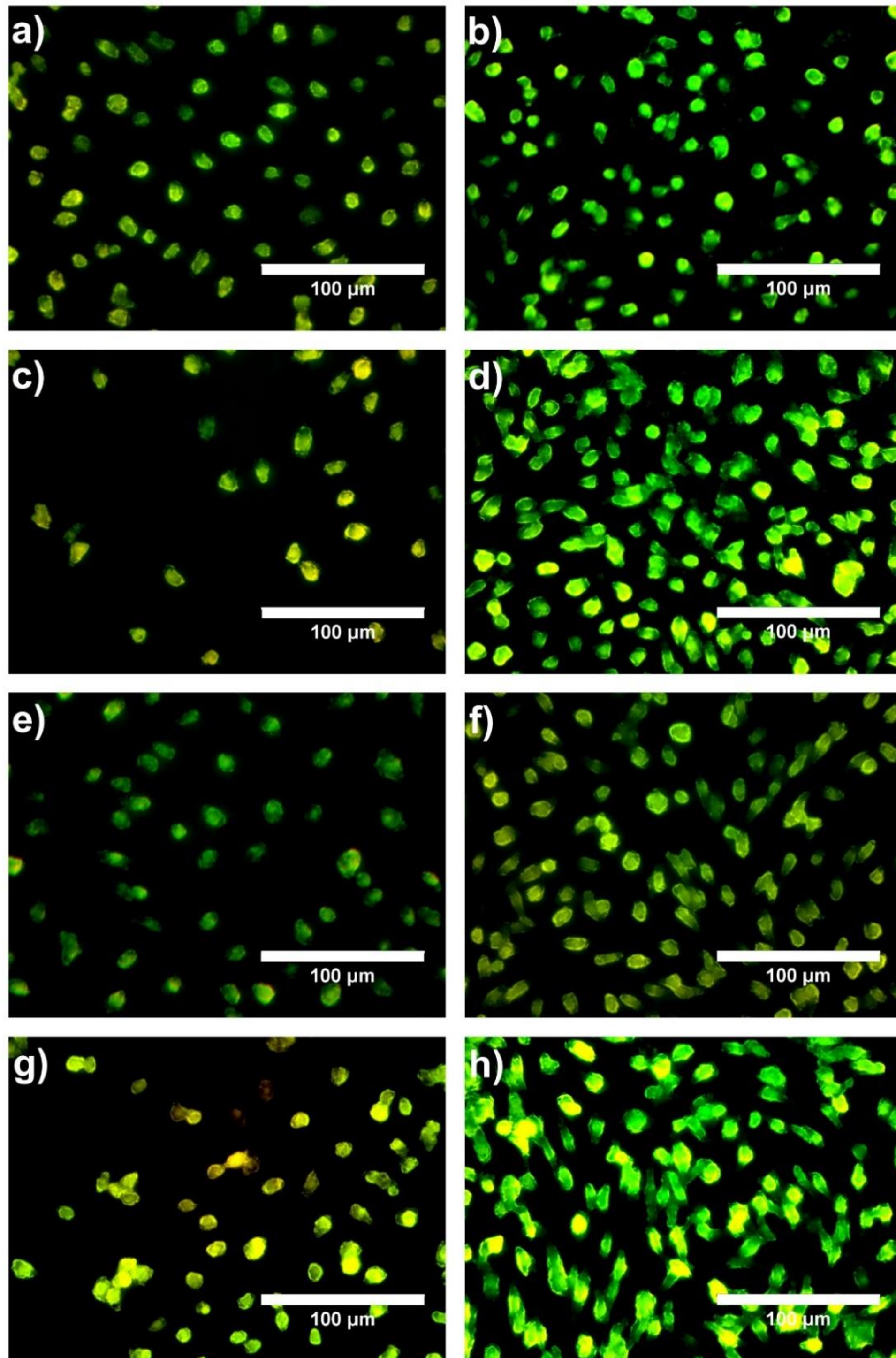

Figure 4.7 Florescence Images of **a)** SC scaffold on 3rd day, **b)** SC scaffold on 7th day, **c)** SC E scaffold on 3rd day, **d)** SC E scaffold on 7th day, **e)** SC EP scaffold solvent cast regions on 3rd day, **f)** SC EP scaffold solvent cast regions on 7th day, **g)** SC EP scaffold electrospun patterns regions on 3rd day, **h)** SC EP scaffold electrospun patterns regions on 7th day (all scales are same and shows 100 µm).

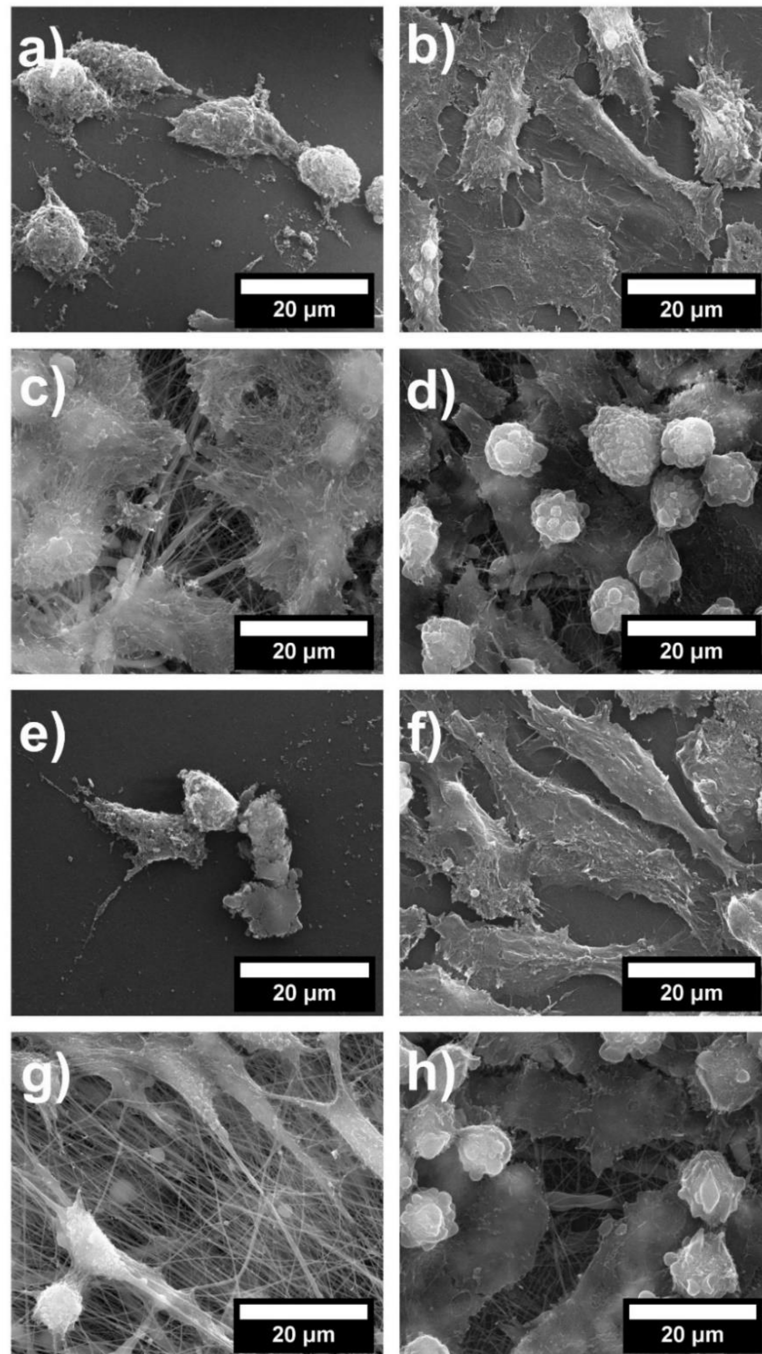

Figure 4.8 SEM Images of **a)** SC scaffold on 3rd day, **b)** SC scaffold on 7th day, **c)** SC E scaffold on 3rd day, **d)** SC E scaffold on 7th day, **e)** SC EP scaffold solvent cast regions on 3rd day, **f)** SC EP scaffold solvent cast regions on 7th day, **g)** SC EP scaffold electrospun patterns regions on 3rd day, **h)** SC EP scaffold electrospun patterns regions on 7th day (all scales are same and shows 20 µm).

CHAPTER 5

Conclusions

- The 2D PCL scaffolds were prepared by solvent casting procedure. PCL (7.5 w/w%) was dissolved in dichloromethane by magnetically stirring for 2 h at room temperature. The solutions in 10 ml volume were poured into 9 cm petri dishes and placed on a flat surface. The samples were kept for 2 days at room temperature for slow evaporation. At the end of 2 days the samples are put into 40°C heated oven to dry for 17 h. Finally, the dried PCL solvent cast samples are kept in room temperature for additional 3 days in order to get rid of residual solvents.
- For electrospininng processes the solution parameters for electrospinning of PCL were adopted from previous works in our laboratory. Initially, PCL pellets were weighted (15% (wt./vol.)) carefully and dissolved in a chloroform and methanol mixture with the 3:1 volume ratio at 30°C. The solution was mixed by magnetic stirrer for 3 hours until clear homogenized solution was observed. Finally, the PCL solution was kept in room temperature for 10 minutes to eliminate air bubbles present in the solution. The electrospining parameters were found experimentally as; i) electrospinning parameters of PCL onto solvent cast without stencil (SC E), collector to needle distance of 17 cm, flow rate of 12 µL/min and 18 kV applied voltage. ii) Electrospinning parameters of PCL onto solvent cast with polyester stencil (SC EP), collector to needle distance of 20 cm, flow rate of 15 µL/min and 20 kV applied voltage. The produced scaffolds are named as; solvent cast film (SC), electrospun fibers onto solvent cast (SC E), electrospun patterns onto solvent cast film (SC EP).
- The average diameter of the circular electrospun patterns were calculated as 4.4±0.6 mm and the area fraction of patterns were found as 41.23% via ImageJ software.

- According to thickness measurements, the thicknesses of SC was found as 169.6±5 µm, SC E was found as 249.4±18 µm, SC EP patterns were found as 212±11 µm and SC EP non-patterned regions were found as 170.6±5 µm.
- SEM imaging of the electrospun fibers revealed bead free and continuous fiber structures for SC E and SC EP. Also smooth nonporous surface for SC scaffolds were obtained according to SEM images. The average fiber diameters of SC E and SC EP electrospun fibers were; 182±51 nm and 186±50 nm, respectively. Also for SC E, average pore size was 1.310 µm and porosity was 39% while for SC EP average pore size was 1.298 µm and porosity was 45%.
- According to contact angle measurements the CA (°) values of the solvent cast scaffold surface has the moderate CA value of ~59°. On the other hand, the electrospun parts of the SC E and SC EP had the CA values of ~110° and ~85° respectively. The difference between the CA (°) values of SC EP and SC E may have resulted from the thinner layer of fibers in SC EP and more porous structure of it when compared to the SC E. Therefore, contact angle values of SC EP fibers were lies between SC and SC E contact angle values.
- The FTIR analyses of the produced scaffolds display the all characteristic peaks of PCL including the characteristic peak of the carbonyl group located at the 1721 cm^{-1}. From FTIR spectra, it can be seen that there was not other material other than PCL in the produced structures.
- The mechanical testing showed that the produced SC scaffolds had brittle properties. And since types of the scaffolds have SC film as the base layer, these brittleness dominates all three samples. Also, SC E scaffold whose surface is entirely consists of electrospun PCL fibers had slightly higher tensile strength and strain at break percentage and lower elastic modulus value when compared to the others.
- The cell culture studies were conducted with mouse fibroblast (L929) cells for 7 days period. Cell attachment assay revealed early attachment tendency of fibroblasts for choosing smooth surfaces over rough surfaces.
- The MTT assay were done and showed that the produced scaffolds enhance the cell attachment, proliferation and differentiation. SC scaffold gave the best performance for the cells on the 1st and 3rd days. This could be as a result of its

low CA (°) value and smooth surface. The absorbance values of all three samples were similar to each other at 5th day and at the last day of the cell culture SC E scaffolds had the highest absorbance values because of its 3D porous fiber structure. Since the SC EP had the area fraction of the circular patterns as 41.23%, its absorbance value stands between SC and SC EP for 1st, 3rd and 7th days. On 3rd day, it is thought that the SC EP absorbance value affected positively by solvent cast parts and negatively by electrospun regions. However, on the 7th day, it can be said that the electrospun patterns were affected the absorbance value positively while solvent cast parts performed poorly when compared to the e-spun regions.

- The florescence microscopy analyses were done to monitor the cell attachment and proliferation and the results were parallel to MTT assay findings. Also, when looked at the SC EP florescence images, it can be seen that the solvent cast parts were similar to SC while its electrospun parts were similar to SC E. Thus, it can be said different cell densities were present at the patterns and solvent cast regions of SC EP for 3rd and 7th days of cell culture.
- SEM imaging micrographs support the MTT assay and fluorescence microscopy results. They show the attachment and the morphologies of the fibroblast cells onto the fibrous electrospun and smooth solvent cast surfaces on 3rd and 7th days of cell culture.
- As a result of the all characterization studies, it was concluded that the direct writing of patterns with a mask by electrospinning is an easy and a promising tool for tissue engineering studies. Also with this method, different production routes of a material can be merged together, thus it is a powerful candidate for various kinds of cell guidance studies for biomedical applications.

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