OSTEOGENIC DIFFERENTIATION OF MESENCHYMAL STEM CELLS ON DOUBLE LAYER ELECTROSPUN SCAFFOLDS

by

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ABSTRACT

OSTEOGENIC DIFFERENTIATION OF MESENCHYMAL STEM CELLS ON DOUBLE LAYER ELECTROSPUN SCAFFOLDS

Bone is the main component of the skeletal system in vertebrates. It provides mobility, locomotion and mechanically supports the soft tissues. Bone related diseases and injuries are generally caused by trauma, genetic malformations, bone loss/overgrow, infections and tumors. While simple fractures are easy to treat, complex fractures and pathological disorders entail arduous treatments. Current orthopaedic solutions encompass invasive surgical procedures and bone grafting (autografts, allografts and xenografts). Despite the fact that they are approved and commonly used methods, they have some unignorable disadvantages. Scientists sought alternative ways to overcome these drawbacks and conclude that tissue engineering could be promising treatment method for bone injuries. In bone tissue engineering, the scaffolds are combined with cells and biological stimulators to favor osteoconduction and osteoinduction during repair and regeneration of damaged or diseased bone.

In this study, PCL (poly-ε-caprolactone)/PLLA (poly L-lactic acid) double layer nanofibrous electrospun scaffold system was designed to mimic natural extracellular matrix (ECM) of 3-D bone tissue. Bone morphogenetic protein-2 (BMP-2) loaded gelatin microspheres were embedded between two layers of electrospun scaffolds to favor osteogenic differentiation. In order to test the system, human adipose derived mesenchymal stem cells (hADMSCs) were seeded on the scaffolds. MTS cell viability assay showed that the double layer scaffold system supported the attachment and proliferation of the cells. The osteoinductive capacity of double layer scaffold system has been proven by alkaline phosphatase (ALP) assay, Von-Kossa staining and confocal microscopy analysis

The result of these studies demonstrated that our scaffold system was suitable for the attachment, proliferation, differentiation and maturation of the hADMSCs.

ÖZET

ÇİFT KATMAN ELEKTRO EĞİRİLMİŞ İSKELELER ÜZERİNDEKİ MEZANKİMAL KÖK HÜCRELERİNİN OSTEOJENİK FARKLILAŞMASI

Kemik, omurgalılarda iskelet sistemini oluşturan dokulardan biri olup hareketliliği, lokomosyonu sağlar ve yumuşak dokulara destek olur. Kemikle ilgili hastalıklara ve zedelenmelere travma, genetik bozukluklar, kemik kaybı/fazla büyümesi, enfeksiyonlar ve tümörler sebep olur. Basit kırıkların tedavisi kolay olurken, kompleks kırıklar ve patolojik hastalıklar zorlu tedaviler gerektirmektedir. Günümüzde kullanımda olan ortopedik çözümler invaziv cerrahi yöntemler ve kemik greftleridir (otogreft, allogreft, heterograft), Bunlar yaygın olarak kullanılan ve onaylanmış tedavi yöntemleri olmalarına rağmen gözardı edilemeyecek dezavantajlara sahiptirler. Bilim insanları, kullanılan yöntemlerdeki eksikliklerin üstesinden gelebilmek için alternatif yollar aramış ve kemik doku mühendisliğinin kemik hastalıklarının tedavisi için gelecek vaadeden bir alan olabileceğinde karar kılmışlardır. Kemik doku mühendisliğinde, hasarlı veya hastalıklı kemik dokusunun onarımı ve rejenerasyonu esnasında osteokondüksiyonu ve osteoindüksiyonu desteklemek amacıyla iskeletler, hücreler ve biyolojik uyaranlarla birleştirilmiştir.

Bu çalışmada, üç boyutlu kemik dokunun ekstrasellüler matriksini taklit etmek amacıyla, poli-ε-kaprolakton (PCL)/poli(L-laktikasit) (PLLA) çift katmanlı nanolif sistem dizayn edilmiştir. Yapı iskelesi elektro-eğirme yöntemiyle hazırlanmış olup osteojenik farklılaşmayı desteklemek için iki katmanın arasına kemik morfojenik proteini (BMP-2) yüklü jelatin mikro küreler yerleştirilmiştir. Tasarlanan iskele sistemini test etmek üzere insan yağ dokusundan elde edilen mezankimal kök hücreler (hADMSCs) iskelelerin üzerine ekilmiştir. Çift katmanlı iskele sisteminin hücre tutunmasını ve çoğalmasını desteklediği MTS testi ile gösterilmiştir. Alkalen fosfataz testi, Von-Kossa boyaması ve konfokal mikroskop analizleri kullanılarak çift katmanlı iskele sisteminin osteoindüktif kapasitesi kanıtlanmıştır. Bu çalışmalar sonucunda, tasarlamış olduğumuz iskelele sisteminin insan yağ dokusundan elde edilen mezankimal kök hücrelerinin tutunması, çoğalması, farklılaşması ve olgunlaşması için uygun olduğu görülmüştür.

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LIST OF ABBREVIATIONS

ALP	Alkaline Phosphatase
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
DAPI	4',6-diamidino-2-phenylindole
DBM	Demineralized Bone Matrix
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
FACS	Fluorescence-activated Cell Sorting
FGF	Fibroblast Growth Factor
FGFR3	Fibroblast Growth Factor Receptor 3
GRAS	Generally Regarded as Safe
hADMSC	Human Adipose Derived Mesenchymal Stem Cell
IEP	Isoelectric Point
IGF	Insulin-like Growth Factor
IL	Interleukin
M-CSF	Macrophage Colony-stimulating Factor
MSC	Mesenchymal Stem Cell
OPG	Osteoprotegerin
PAH	Poly(allylamine) Hydrochloride
PBS	Phosphate Buffered Saline
PCL	Poly-ε-caprolactone
PDGF	Platelet-derived Growth Factor
PDLA	Poly D-lactic acid
PGA	Polyglycolic Acid
PLA	Polylactic Acid
PLCL/HA/Lam	Poly (L-lactide-co-ε-caprolactone)/hydroxyapatite/laminin
PLDLA	Poly-L/D-lactic Acid

PLGA	Polylactic-coglycolic Acid
PLLA	Poly L-lactic Acid
p-NPP	P-nitrophenol Phosphate
PSS	Poly Sodium 4-styrenesulfonate
PTH	Parathyroid Hormone
RANK	Receptor Activator of Nuclear Factor
RANKL	Receptor Activator of Nuclear Factor κβ Ligand
RGD	Arg-Gly-Asp
ROP	Ring-opening Polymerization
SEM	Scanning Electron Microscopy
TGF	Transforming Growth Factor
UV	Ultra Violet

1. INTRODUCTION

Tissue engineering is a developing field since mid-1960s that intends to construct a functional and feasible alternates to damaged or diseased tissues and organs [1, 2]. When conventional medical strategies became insufficient for the treatment of injured tissues and organs, researchers came up with the idea of tissue engineering. It is a multidisciplinary research area coalescing the knowledge, experience, principles and methods of biology, material science, chemistry, physics and engineering [3]. The major components of the tissue engineering are 3D scaffolds, cells and biological stimulators. Selection of the each component varies depending on the chemical, mechanical, biological and structural characteristics of the tissue which is designated to be study on.

3D scaffolds are used to create artificial extracellular matrices (ECMs) for cells to attach, proliferate, migrate and differentiate. They should have a porous structure that allows transportation of nutrients, metabolic wastes and growth factors. They should also be non-immunogenic, biocompatible and biodegradable with an optimal degradation rate to support cell-tissue growth and maturation [4, 5].

Bone tissue originated disorders, such as bone fractures-injuries, musculoskeletal diseases, osteoporosis, and tumors etc., affect millions of people all around the world. This situation is caused by both increasing median age of the population and vulnerability of the skeletal system to outside impacts [6]. Bone tissue engineering aims to reconstruct damaged tissues by using bioengineering principles. Reconstruction of the bone tissue can be accomplished by two basic methods (Figure 1.1). The first method includes only scaffold systems without cells to favor bone ingrowth (osteoconduction). The empty scaffolds fill the gaps between cells and support them mechanically. The second method combines cells (stem cells, progenitor cells etc.) with the scaffold systems to support osteogenesis (direct bone formation), osteoinduction (differentiation to skeletal cells from non-skeletal cells) besides osteoconduction [7]. The method of combination of cells with scaffolds is more preferable to study *in vitro* than the first one, since it is thought to be more advantageous in terms of bone remodeling.

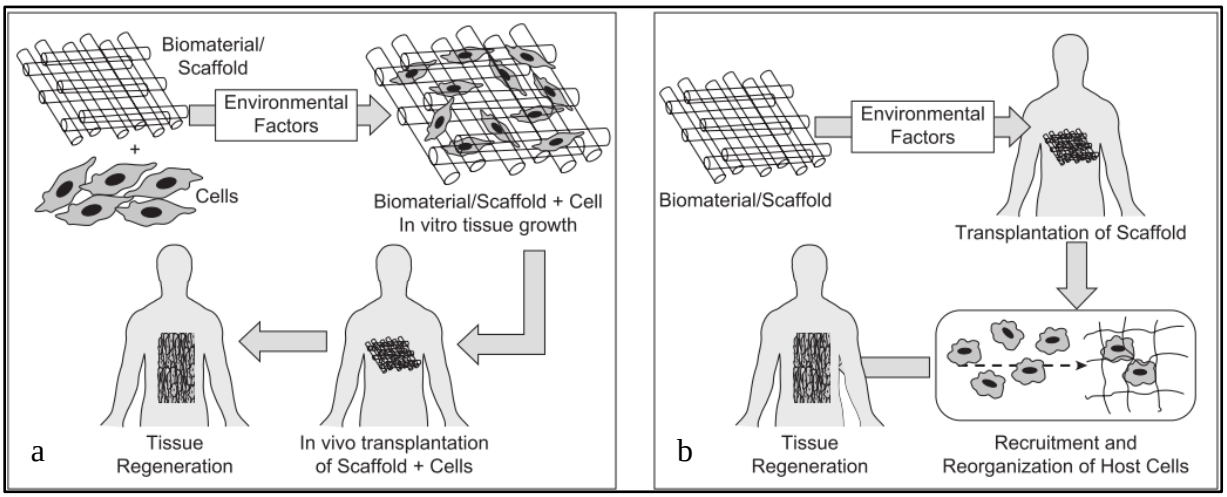

Figure 1.1. The two basic approaches for bone tissue engineering: (a) transplantation of cell-seeded scaffolds that are constructed *in vitro*, (b) providing bone tissue regeneration *in vivo* with the transplantation of empty scaffold [8].

2. THEORITICAL BACKGROUND

2.1. BONE PHYSIOLOGY

Bone is the major component of the adult skeletal system in vertebrates. In human body, bone mechanically supports the lung expansion and the movement by enabling effective muscle contraction. It also protects internal soft tissues and organs particularly brain, heart, liver and stomach [9, 10].

Bone is consisting of both organic and inorganic substances. The organic part of bone is composed of proteins collagen type I (~90-95 %) and a small amount of collagen type V. The noncollagenous proteins of the organic portion are osteopontin, thrombospondin and fibronectin (cell adherence/signalling proteins): biglycan and decorin (proteoglycans); proteins linked with calcium such as osteocalcin and matrix gla protein; and bone morphogenetic proteins (BMPs). Besides some bone non-specific proteins are also exist in the organic part of bone such as osteonectin and osteopontin [11]. Hydroxyapatite crystalline ($Ca_{10}(PO_4)_6(OH)_2$) together with magnesium, sodium, carbonate and fluoride minerals forms the inorganic compartment of the bone. The rigidity, hardness and compressive strength of the bone are provided by these crystalline structures. Contrariwise, bone collagen fibrils have low compressive strength, high elasticity and good tensile strength. The combination of the mechanical properties of both organic and inorganic parts helps bone to resist mechanical stresses [10].

Bone possesses three types of cells; osteoblasts, osteocytes and osteoclasts. Additionally, osteoprogenitor cells also exist in bone. These cells are mesenchymal stem cells that differentiate into osteoblasts and osteoclasts. While osteoblast precursor cells are originated from mesenchyme, osteoclast precursors are generated by blood monocytes [12].

Osteoblasts are the major cells that are responsible for bone formation. Organic elements of bone matrix, such as collagen type I, glycoproteins and proteoglycans, are produced and

secreted by osteoblasts. The newly formed osteoblasts (derived from mesenchymal osteoblast progenitor cells) are located at the top of developing bone tissue and have cuboidal shape with basophilic cytoplasm (Figure 2.1). They briskly synthesize the bone matrix. When their synthesizing activity decreases, they exhibit narrower, longer and flatter morphology and become bone lining cells that present in endosteum and periosteum [13, 14].

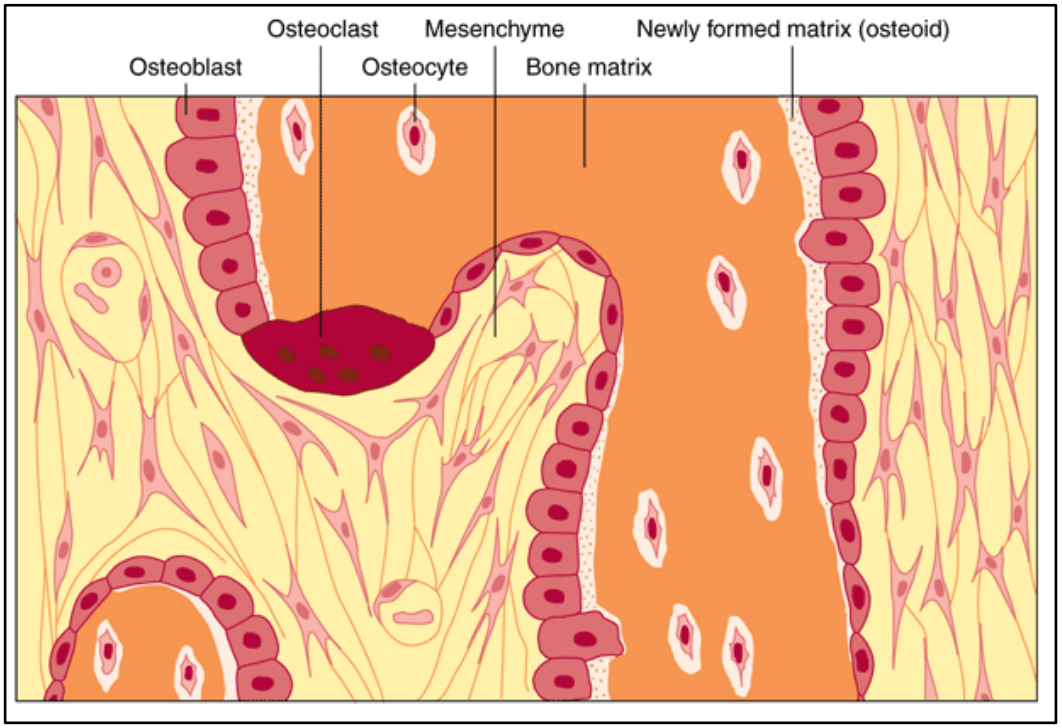

Figure 2.1. Schematic representation of major bone cells [15]

Osteoblasts synthesize unmineralized matrix material (osteoid) during new bone formation. The process of mineralization is initiated after the formation of osteoid. Vitamin K-dependent protein osteocalcin and glycoproteins (produced by osteoblasts) raise local calcium concentration by binding Ca^{2+} ions. Meanwhile, osteoblasts synthesize alkaline phosphatase (ALP) enzyme and release it with some other enzymes in a membrane enclosed matrix vesicles. ALP removes phosphate groups from organic phosphates and increases PO^{4-} ion concentration in the environment. Elevated concentrations of calcium and phosphate ions lead hydroxyapatite crystal formation and accumulation. Continuous accretion of these minerals creates calcified bone matrix and the mineralization process is eventually completed (Figure 2.2) [14, 15].

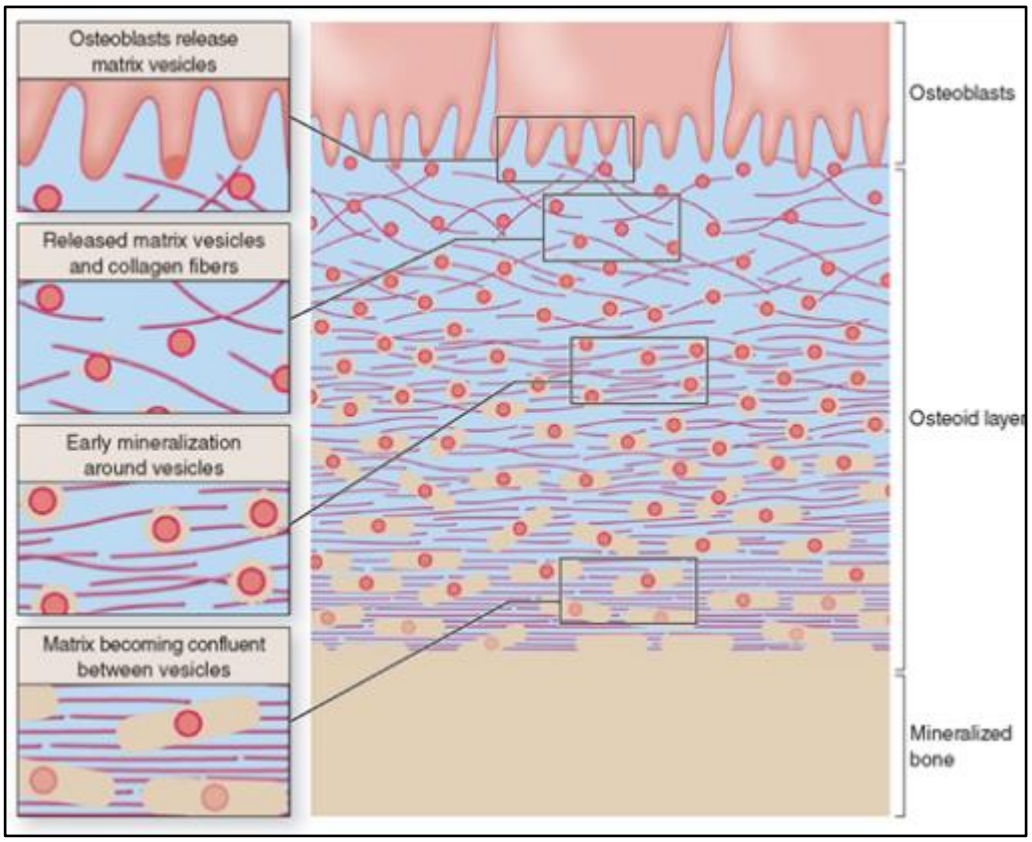

Figure 2.2.Bone matrix mineralization [14]

As the time passes by, osteoblasts are encircled by extracellular matrix (ECM) of bone and undergo some alterations. They develop slender, long processes named filopodia, lose their organelles and turn morphologically into star-like cells. These newly transformed cells are called osteocytes which are located in the small cavities of bone matrix (lacunae). They connect with each other through their filopodia that present into the canaliculi (small channels within the bone matrix). They communicate with each other via gap junctions that exist at the tips of their filopodia. Gap junctions provide nutrient and oxygen transportation, additionally they are also used for cellular waste disposal. The major function of osteocytes is to detect the amount of mechanical stresses and strains at which bone is exposed. They also regulate calcium and phosphate homeostasis. Besides, osteoblasts synthesize a protein; sclerostin, that blocks osteoblastic bone formation and induce osteoclast formation. All of these functions have significant roles in the regulation of bone remodeling [12, 16].

Osteoclasts are big, multinucleated cells that are derived from hematopoietic stem cells. Their production depends on two polypeptides synthesized by osteoblast; RANKL (receptor activator of nuclear factor κβ ligand) and M-CSF (macrophage colony-stimulating factor). Osteoclasts subsist within enzymatically eroded resorption cavities called Howship lacunae. Mature osteoclasts are found in the resorbing surfaces of bone. Subsequent to their activation, they migrate through microfractures of bone, attach via podosomes (actin-rich adhesion structure) and create ruffled border. Then, they release hydrogen ions produced by carbonate dehydratases for the formation of resorption cavities. The crystalline hydroxyapatite is acidified and dissolved within the acidic environment generated by proton translocation from cytoplasm to the resorption area. Furthermore, osteoclasts also secrete collagenase and cathepsin K to digest organic matrix elements of the bone [14, 15, 17].

2.2. BONE DEFECTS AND TREATMENTS

Bone diseases and injuries can be originated from trauma, bone loss/overgrow infections or hereditary malformations. They might also be age-dependent or developed as a consequence of another disorder.

Bone fractures are generally formed as a result of traumatic injuries. Most of the fractures are recovered naturally since bone has a good repair capacity due to the presence of osteopregenitor stem cells and blood vessels [14]. Fracture healing is basically consisted of four stages: hematoma formation, cartilage formation (soft callus), bone formation (hard callus) and bone remodeling (Figure 2.3). Disruption of circumambient soft tissues, blood vessels and muscles is followed by formation of hematoma which provides an area for bone repair. Subsequent to hematoma formation, a cellular response is initiated with the inflammatory cells and the mesenchymal stem cells at the site of breakage. Inflammatory cells, especially thrombocytes, synthesize and release cytokines and growth factors that are required for angiogenesis, proliferation, differentiation of osteoprogenitor cells and cellular chemotaxis. Platelet-derived growth factor (PDGF) is responsible for mitogenesis, induction of angiogenesis and recruitment of inflammatory cells. Interleukin (IL)-1 and IL-6 are also needed for the accumulation of inflammatory cells at the fracture site.

Meanwhile, the mesenchymal stem cells (mostly reside in periosteum) differentiate into chondrocytes and osteoblasts. The differentiation of mesenchymal stem cells into osteoblasts is directed by bone morphogenetic proteins (BMPs). Osteogenic and chondrogenic differentiation of mesenchymal stem cells are affected by opposite conditions; while movement, low oxygen and low pH promote chondrogenic differentiation, immobilization, high oxygen tension and high pH incline toward osteogenic differentiation. In the second phase of fracture healing, a semi-rigid soft callus is formed by fibroblasts and chondrocytes. The newly formed soft callus mechanically supports the fracture and acts as a formwork for hard callus. The soft callus is gradually replaced by hard callus in a process called endochondral ossification. Chondrocytes within the primary center of ossification terminate expressing collagen type II and proteoglycans and in the meantime osteoblasts start secreting alkaline phosphatase for mineralization. Mineralization leads to chondrocyte degeneration, hypertrophy and apoptosis. After the woven bone formation and further mineralization, hard callus is produced around the broken ends of bone. Finally, the woven bone hard callus is remodeled by turning it into the original cortical/trabecular bone composition [18, 19].

Osteoporosis and osteomalacia are two basic diseases characterized with low bone mass [20]. Osteoporosis is a condition described by decreased bone mass which makes a person susceptible to fractures with a minimal trauma [21]. It is classified as primary and secondary osteoporosis. Primary osteoporosis is an age-related situation that develops in postmenopausal women or in older men/women. In postmenopausal osteoporosis, the deficiency of estrogen hastens bone mass loss since estrogen increases the activity of osteoclasts without increasing osteoblastic activity. Estrogen deprivation promotes release of cytokines IL-6, transforming growth factor (TGF) and colony stimulating factor (M-CSF); which all together induce the proliferation of osteoclast progenitor cells. Meantime RANKL is produced by osteoblasts and binds to its receptor found on osteoclasts. RANKL possess an antagonist osteoprotegerin (OPG) which is a soluble receptor that is synthesized by the stromal osteoblast lineage cells. Besides, its production is stimulated by estrogen. The RANK-RANKL-OPG molecular triad regulates the osteoclast production signals. The decreased estrogen level results in low secretion of OPG, thus RANKL binds RANK and increases the number of osteoclasts. Consequently, osteoclasts favor the bone resorption and cause postmenopausal osteoporosis [22, 23].

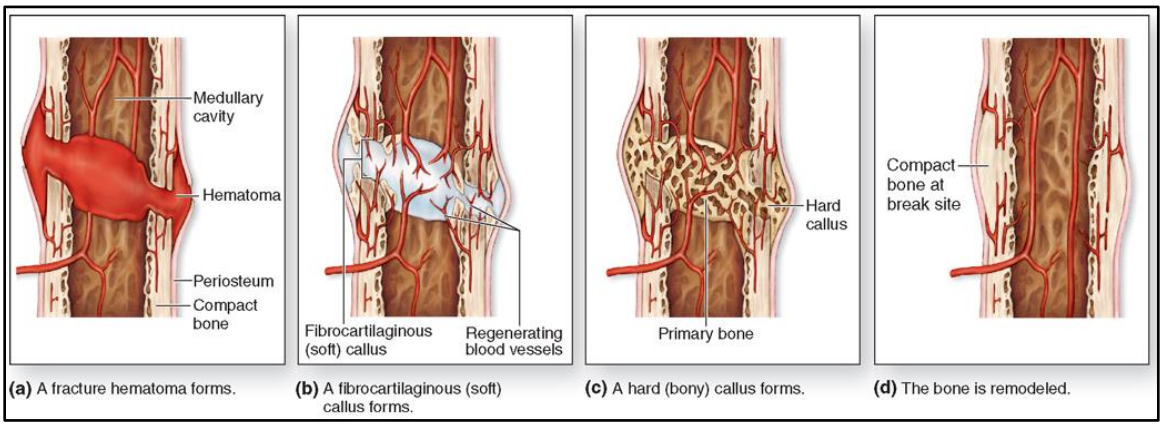

Figure 2.3. Main steps of bone fracture repair [14].

Renal and intestinal functions become atrophic with the increasing age of human. The inadequate production of vitamin D and a decline in capability of the kidney to convert 25(OH)D to 1,25(OH)2D cause less efficient calcium absorption in intestines. This condition leads to reduction in plasma calcium concentration and excessive secretion of parathyroid hormone (PTH) (indirectly induces osteoclasts to elevate blood calcium levels). The hypersecretion of PTH causes accelerated bone loss in osteoporosis [22].

The secondary osteoporosis occurs as a result of other clinical disorders such as endocrine disorders (hyperparathyroidism, hypogonadism etc.), gastrointestinal diseases (Vitamin D deficiency, chronic liver disease, malabsorption etc.), drugs, and immobilization. The treatment methods of osteoporosis include vitamin D-calcium supplementation, pharmacological molecules and drugs such as denosumab (human monoclonal antibody that inhibits RANKL), bisphosphonates (inhibit osteoclast function and reduce bone resorption), selective estrogen receptor modulators (raloxifene [Evista]) and calcitonin [22, 24, 25].

Osteomalacia is a disorder characterized by malformed mineralization of bone, resulting in accretion of osteoid (unmineralized bone matrix). In children, this mineralization defect occurs in growing bone, before epiphysis closure, and it is named as rickets. Osteomalacia is generally arise from vitamin D deficiency and infrequently developed contingent upon hypophosphataemia. Vitamin D is a prohormone that promotes osteoblasts for production

of mineralized bone matrix and increases calcium absorption. Therefore low vitamin D level is associated with reduced bone/matrix ratio and calcification which predispose bone to fractures [22, 26, 27]. Calcium and vitamin D supplementations are needed for the treatment of osteomalacia and rickets diseases [25, 28].

The most common inherited bone diseases are osteogenesis imperfecta, achondroplasia, and osteopetrosis. Osteogenesis imperfecta is a heritable disorder that classified into several different types according to the mutations they possess. The incidence of the disease is nearly one in 10,000 births [29]. Mutations in the genes of COL1A1 or COL1A2 that encode respectively the α1 and α2 chains of type I collagen cause qualitatively defected or quantitatively decreased collagen fibrils. Due to the deficient fibrils, bones become fragile and have low bone mass [30].

Achondroplasia is an autosomal dominant disorder caused by a mutation in the type 3 receptor for fibroblast growth factor (*FGFR3*) encoding gene. The mutation affects many types of tissues, especially the cartilaginous growth zone in the growing skeleton. The disease is the primary cause of the 70% of cases of dwarfism and phenotypically characterized by enlarged head, short hands and valgus legs [31-33].

Osteopetrosis is a group of uncommon inherited disorders that affect skeletal development because of a defect in the bone resorption mechanism due to osteoclast dysfunction [25, 31]. Paget disease is another type of bone disorder that is also characterized by abnormal bone remodeling resulting from excessive activation of osteoblasts and osteoclasts [25].

Besides these diseases, bone tumors (multiple myeloma, osteosarcoma etc.) and infections (osteomyelitis) also cause bone malformations.

Although bone has high self-repair capacity, large bone defects and non-unions caused by bone defects require orthopaedic treatments and surgical interventions. Bone grafting is the most common procedure that is used for the treatment of critical-size bone defects. Bone grafts are categorized into three groups: autografts, allografts and xenografts. The bone grafts transplanted within the same individual are defined as autografts. In this treatment, a part of the bone is transplanted from one site of the body to another (generally from iliac

crest or pelvis) to fill the defect of the bone. Autografts are referred to as the current gold standard treatment method since they possess both osteoinductivity and osteoconductivity. They stimulate the mesenchymal stem cell recruitment to the grafted area and mesenchymal stem cell differentiation into osteoblasts. In spite of these advantageous properties, autogenous bone grafting has major drawbacks such as donor site morbidity, scarce availability of bone grafts, pain, prolonged hospitalization period and increased risk of infection and inflammation due to additional surgery. Therefore bone graft alternatives, allografts and xenografts have been developed [34-36].

Allografts surmount the limitations of autologous bone grafting. They have no donor site morbidity. Since they are readily available, there is no time consumption. They can be altered to be a composite and used in large defects since the amount of graft is not limited as the autograft is. However, there is a risk of pathogenicity, immunogenicity and the products vary since allografts obtained from bones of cadavers or from living donors harvested during orthopedic surgeries. Most of the allografts consist of freeze-dried, heat-treated and frozen bone to overcome immunological reactions but these treatments result in a reduced osteoinductive activity (cells are not alive and growth factors are partly inactivated). For this reason, allogenic materials were improved and new products were generated such as demineralized bone matrix (DBM), which is derived by acid extraction of human cortical bone grafts. It includes cell residue, growth factors such as BMPs, extracellular matrix and minerals [35, 37, 38].

Xenografts are bone materials obtained from non-human species. Although former achievements in xeno-transplantation draw researchers' attention towards this approach, it is now considered to be inappropriate for bone grafting due to the host rejection, risk of infection, disease transmission and toxicity [18].

Additionally, stainless steel, titanium, screws and pins can be used in operative fracture fixation, which requires high mechanical strength and flexibility of the fixative materials [3].

Bone tissue engineering was developed as an alternative treatment way to bone grafts and other therapies. It combines osteoprogenitor cells and osteogenic stimulators with scaffolds and favors bone tissue regeneration [39].

2.3. BONE TISSUE ENGINEERING SCAFFOLDS

Bone tissue engineering scaffolds are designed to imitate natural characteristics of bone ECM to support cell attachment and new tissue formation. The mechanical strength, stiffness and elasticity are incontrovertibly important for bone tissue in order to protect internal organs, to bear large forces and maintain musculoskeletal locomotion. Elasticity and stiffness of the materials are determined by the Young`s Modulus (modulus of elasticity) value. It measures the endurance of an object to elastic deformation under two contrary longitudinal forces, in another sense, it can be described as the tendency of a material to get back into its original position after a force has been applied. Mathematically it is denoted as the ratio of tensile stress to tensile strain. The magnitude of the Young`s Modulus is directly proportional to stiffness, meaning that the higher the Young`s Modulus, the stiffer the material is [40, 41]. Therefore, the scaffolds that will be used for bone tissue engineering should be designed considering all of these mechanical features of the bone. The Young`s Modulus value intervals for human bone structural elements are shown in Table 2.1.

Table 2.1. Young`s Modulus values of the human bone structural elements [42, 43].

	Young`s Modulus, E (GPa)
Cortical Bone (Longitudinal)	11 – 21
Cortical Bone (Transverse)	5 – 13
Cancellous Bone	0.05 - 0.5
Bone Mineral (Hydroxyapatite)	80
Collagen (Dry)	6

The scaffolds must be made of the material which is biocompatible with the human body. This is essential because if the material is not biocompatible, it can generate cytotoxic degradation products or trigger an inflammatory response that could lead undesirable consequences [44]. Biodegradability at a proportionate rate with the new bone formation, osteoconductivity and osteoinductivity are the other criteria that a bone scaffold should certainly meet [7]. Additionally, permeability of the bone scaffolds is a crucial feature because it is required for cell migration, cell proliferation, vascularization, oxygen diffusion, nutritional fluid flow and transportation. Permeability hinges upon porosity, pore diameter, and the shape of the pore. The optimal porosity is 90 % for bone constructs to conserve mechanical and structural stability. The minimal suggested pore size for bone scaffolds is 100 μm, which is sufficient for cell migration, exchange of nutrition and metabolic waste, however bone scaffolds with pores > 300 μm have indicated better osteogenesis since they permit vascularization and therefore higher oxygenation [44, 45]. Furthermore, it has been proved that smaller pore size and porosity cause chondrogenesis [46].

Bone scaffold materials are generally categorized into two groups; natural polymers and synthetic polymers. They can be interchangeably used for bone tissue engineering with regard to their structural properties. Combining them with the cells and signaling molecules reinforces the construction of new bone (Figure 2.4).

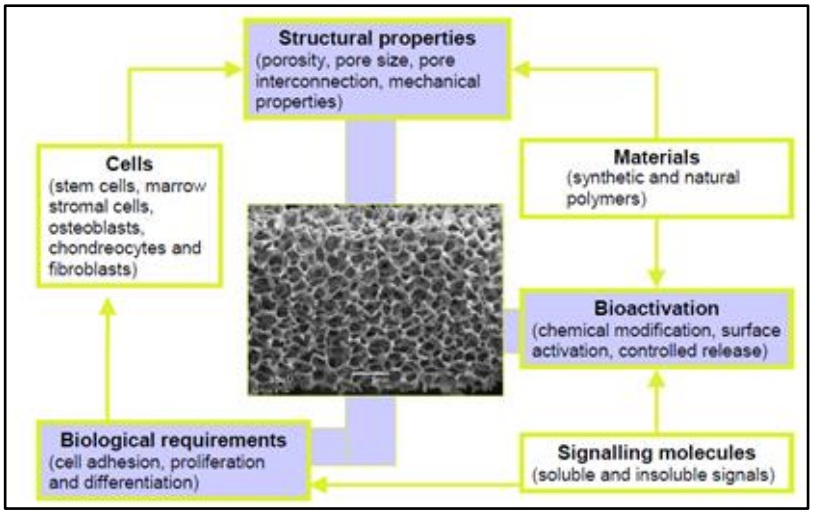

Figure 2.4. Important factors required for the design of the ideal bone tissue engineering scaffold [47].

2.3.1. Natural Polymers

The most abundantly used natural polymers in bone tissue engineering are collagen, gelatin, alginate, chitosan, silk, fibrin and hyaluronic acid. In recent years, natural-origin polymers have attracted the attention of scientists due to their biodegradability, biocompatibility, low toxicity, renewability and profitability (low manufacture and disposal costs). Besides these advantageous properties of the polymers, the natural biomaterials can be degraded by cells with proteolysis; furthermore, they promote cell adhesion and bone remodeling. In spite of these favorable features, natural polymers have some drawbacks such as mechanical weakness, low thermal and chemical stability, immunogenicity and risk of infection by pathogens [4, 8, 48]. Rapid degradation of natural materials is an obstruction for bone tissue engineering since mechanical stability is crucial. Cross linkers such as gluteraldehyde, genipin or synthetic biomaterials (which can strengthen the natural polymers mechanically) might overcome this problem.

Collagen, the major protein of the mammalian connective tissue, is found in the structure of skin, tendons, bones, cartilage, blood vessels, and ligaments. Collagen type I is the most widespread and investigated fibrous protein among twenty-seven types of collagen. As an ECM element, collagen interacts with all cells in tissues. The cell attachment, migration

and proliferation are guided by the vital signals provided by collagen. Collagen is a biocompatible material but it displays poor mechanical properties. The degradation rate of collagen is hard to control, so it must be processed or blended with other polymers in order to be used as a scaffold for tissue engineering applications. It can be cross-linked chemically using polyepoxy, aldehydes carbodiimides etc., or physically (such as dehydrothermal treatment, ultraviolet/gamma/microwave irradiation) to elevate mechanical features (by enhancing tensile strength of collagen fibers) and to maintain chemical stability (by reducing collagen solubility and making it more resistant to the enzymatic degradation) [49-51].

Gelatin is a biopolymer that has been produced by denaturation of collagen by partial hydrolysis. It carries both cationic and anionic groups with some hydrophobic extensions. Lysine and arginine form ~ 13 % positively charged part of the gelatin molecule, ~ 12 % of the molecule is negatively charged (glutamic and aspartic acid) and hydrophobic chain includes leucine, isoleucine, methionine, valine (~11%). The rest of the molecule contains glycine, proline and hydroxyproline (Figure 2.5) [52]. Gelatin has many integrin-binding sites such as Arg-Gly-Asp (RGD). This amino acid sequences provide cell anchorage, cell recognition, proliferation and differentiation. Cell-cell communication and cell-matrix interaction are dependent on integrin receptors.

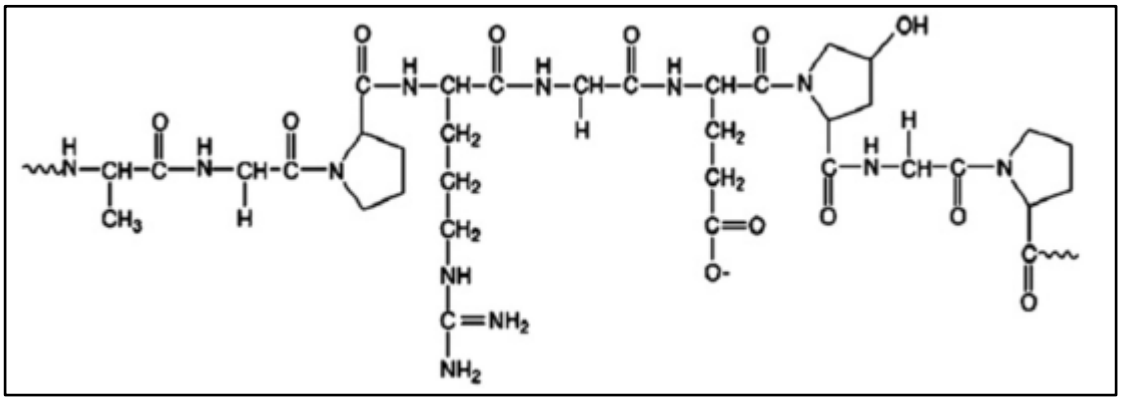

Figure 2.5.The chemical structure of gelatin [52].

The convergence of collagen into gelatin requires pre-treatment. At first, collagen should be turned into a form convenient for the warm-water extraction process (heating collagen in water at a temperature > 45 ℃). This procedure is done for the cleavage of hydrogen and covalent bonds. Subsequent to heat treatment, a chemical pre-treatment should be done in order to rupture non-covalent bonds. The type of the pre-treatment method determines the type of gelatin (type A and type B) obtained from them. For instance, if the collagen is exposed to an acidic treatment, the isoelectric point (IEP) of the gelatin will increase (IEP~9.0), resulting basic gelatin (type A). Conversely, the alkaline treatment yields negatively charged gelatin, which reduces the IEP (~ 5.0) and produces acidic gelatin (type B). This situation plays an important role in the use of gelatin as a carrier system. If a protein is acidic, it should be carried in a basic gelatin or vice versa. Gelatin carrier systems can be fabricated as hydrogels, film or microspheres. These systems can be used as controlled-release devices to encapsulate growth factors like BMP-2, transforming growth factor (TGF-β1, TGF-β2) or fibroblast growth factor (FGF) to stimulate new bone formation [52-55].

Furthermore, gelatin has good biocompatibility, biodegradability in physiological environments and water adsorption capacity. These properties make gelatin suitable for pharmaceutical purposes (drug delivery agents etc.) and biomedical applications such as hard tissue engineering, moreover it has been approved by the United States Food and Drug Administration as a GRAS (Generally Regarded As Safe) material [56, 57].

Alginate is an anionic biomaterial isolated from brown algae (Phaeophyceae), and its species including Laminaria hyperborea, Laminaria japonica, Laminaria digitata, Ascophyllum nodosum, and Macrocystispyrifera by water based alkali solutions (NaOH etc.). It is composed of polymerized (1,4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues. Alginate can exist in the form of blocks with repeating M residues (MMMMMM), repeating G residues (GGGGGG) or blocks of blended G-M residues (GMGMGM). These varying forms can change the functional and mechanical properties of alginate. For example, while G blocks construct stiff chains, M-G blocks form flexible chains. In addition, alginate is described as a biocompatible, non-immunogenic and non-toxic biopolymer. Nevertheless, it is non-degradable since mammals do not have the enzyme for the cleavage of polymer chains, but partial oxidation of the alginate chains and

the ionic cross-linking are the methods applied to surmount this problem. Since native alginate destitute of mammalian cell adhesivity, alginate derivatives (ex. introduction of peptide side chains, like RGD sequence, to stimulate and arrange cellular interactions and anchorage) are used for cell encapsulation and entrapment. For bone tissue engineering, gelatin can be used in the delivery of proteins, osteoinductive agents and osteogenic cells [58-60].

Chitosan is a linear polysaccharide produced by de-N-acetylation of chitin in the presence of alkaline solution (NaOH) or chitin deacetylase (a hydrolytic enzyme). High adsorption capacity, biocompatibility, biodegradability, flexibility, porosity and non-toxicity are the advantages of chitosan. On the other hand, it has some handicaps like mechanical weakness and instability. Blending chitosan with hydroxyapatite, bioceramics or bioglasses improves its mechanical properties. Improved chitosan can be used in tissue engineering applications, drug delivery and gene delivery. It can be processed into hydrogels, nanofibers, micro/nanoparticles, beads and sponges [61-64].

Silk is a protein polymer obtained from variable insects and spiders. Cocoon silk from the silkworm *Bombyxmori* and the silk from the spider *Nephilaclavipes* are the most common types of silk that are used in tissue engineering applications. Sericin and fibroin are the two major proteins found in the structure of silkworm silk. Distinctively spider silks are composed of major ampullatespidroins, minor ampullate-like spidroin, glycoproteins and lipids. The varying amino acid sequences in these silk proteins lead different mechanical properties. High flexibility, good load bearing capacity, high tensile strength, arrangeable degradation properties and thermal stability make silk biomaterials convenient for broad range tissue engineering applications [65-68].

Fibrin is a fibrous protein involved in blood coagulation process. In response to an injury, serine protease thrombin initiates the polymerization of the fibrinogen to form fibrin protein. It serves as a scaffold during tissue regeneration by providing physical support to macrophages, neutrophils and by allowing fibroblasts to penetrate through the site of injury. Since fibrin possesses plentiful binding sites for integrins, growth factors and ECM elements such as fibronectin, fibulin, thrompospondin, it can be used as a scaffold for differentiation of stem cells, angiogenesis stimulation and stem cell delivery. Although

fibrin gels are susceptible to be degraded in an uncontrollable manner by proteolytic cleavage (with plasmin or matrix metalloproteinases), degradation rate can be arranged by adding protease inhibitors and blending fibrin with some other materials such as collagen or synthetic polymers. Apart from these, biocompatibility, ease of manipulation and fast polymerization capacity make fibrin a promising scaffold for tissue engineering [69, 70].

Hyaluronic acid (hyaluronan) is a glycosaminoglycan and one of the major elements found in the ECM. It is a linear polysaccharide composed of repeating units of β-1,4-D-glucuronic acid and β-1,3-N-acetyl-D-glucosamine. Hydrophilicity, biocompatibility, biodegradability and bioresorbability of the hyaluronic acid make it a good candidate to be used as a tissue engineering scaffold [71].

2.3.2. Synthetic Polymers

Synthetic biomaterials are extensively utilized for tissue engineering implementations because of their favorable properties. Unlike natural polymers, synthetic polymers can be easily tailored chemically or mechanically according to the desired properties of the tissue scaffold. The processibility of synthetic polymers allows us to arrange duration and degradation characteristics of the polymer (biodegradability), physical properties such as hydrophilicity, porosity and pore diameter, and mechanical properties like tensile strength, modulus of elasticity by controlling conformation and configuration of polymeric chains and molecular weight of the polymers. In addition, they can be treated with functional groups (carboxyl, hydroxyl, amino etc.) or peptide sequences (integrin/heparin binding peptides) to support cell adhesion. Synthetic bone tissue engineering scaffolds include polyesters, polyurethanes, polyanhydrides and polyfumarates [72].

Poly (lactic acid) (PLA and its derivatives PLLA, PDLA, or PDLLA), poly(glycolic acid) (PGA), poly(lactic-coglycolicacid) (PLGA), poly(ε-caprolactone) (PCL) are widely known and investigated polyesters. They are synthesized by two methods: condensation polymerization and ring-opening polymerization (ROP) of cyclic esters. Polymerization with condensation of hydroxyl-acids or mixtures of diacids and diols requires high temperatures and long reaction times. Since the carbon chain length of the polyesters synthesized with condensation reaction is not long enough and the reaction is reversible,

high molecular weight polymers cannot be obtained from this reaction. ROP method is used to produce polyesters with high molecular weight. Lactones glycolide, lactide and ε-caprolactone are generated by the conversion of α-hydroxy acid into the cyclic esters. Then, these lactones can under undergo ROP initiated mostly by metal catalysts to produce predictable and high molecular weight polyesters (Figure 2.6) [72-76].

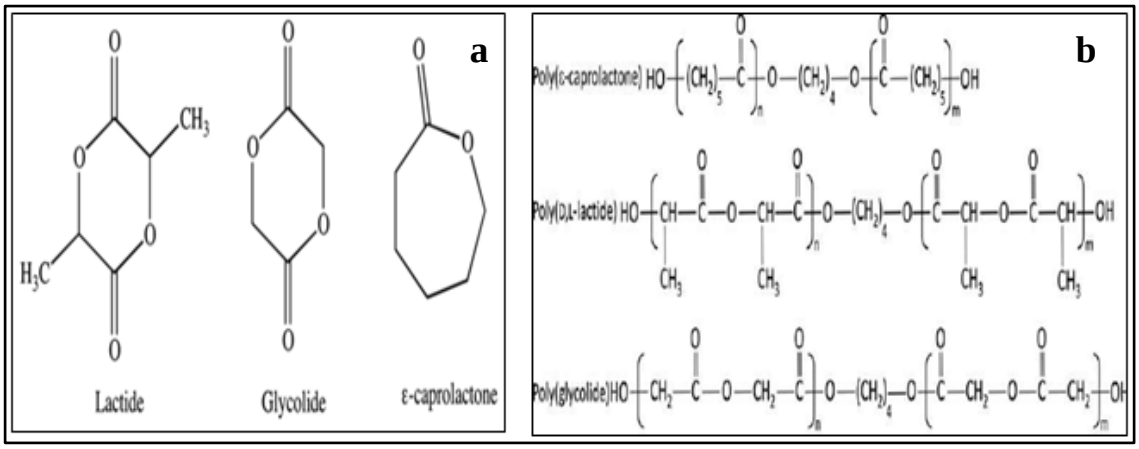

Figure 2.6. (a) Cyclic esters used in ROP [73], (b) polyesters that have been obtained from ROP of common lactones: lactide, glycolide and ε-caprolactone [72].

PCL was synthesized at the beginning of 1930s and has been used in various studies and applications since then. It is a hydrophobic, biocompatible and semi-crystalline material whose crystallinity depends on the molecular weight. PCL has a glass transition temperature (Tg) of − 60℃ and low melting point that varies between 59 and 64℃ both of which properties make the formation of PCL easier at comparatively low temperatures [77].

Bacteria and fungi are able to degrade PCL; however it cannot be degraded rapidly in human or animal bodies because they do not have the suitable enzymes for PCL degradation. In spite of that situation, PCL undergoes two different degradation processes which are non-enzymatic hydrolytic cleavage and enzymatic fragmentation. The non-enzymatic cleavage is initiated in the amorphous reagent of the polymer. The surface degradation or erosion occurs via the polymer backbone scission at the surface. The fragments produced throughout the restriction process become oligomers and monomers, and then diffuse to the environment faster than the entrance of water into the bulk polymer.

Consequently the polymer gets thinner over the time (4-6 months). The length or molecular weight of the fragments for diffusion should be below 5000. When the water enters the complete polymer, leading hydrolysis throughout the whole polymer, bulk degradation occurs. In bulk degradation, hydrolytic chain cleavage occurs randomly and causes a decrease in the molecular weight of the polymer. The enzymatic fragmentation takes place after the production of low molecular weight fragments. Subsequent to phagocytosis of these fragments, intracellular degradation occurs via citric acid cycle. It is proven that the biodegradation rate of PCL depends on the hydrophobicity, crystallinity, length of polymer, presence of enzymes and initial molecular weight of the material [77, 78].

PLA is a thermo-plastic biopolymer synthesized from monomers of renewable sources such as sugar, potatoes and corn. High molecular weight PLA is manufactured by ROP of lactide monomer derived from lactic acid. PLA also exist in several distinct forms which are poly(l-lactic acid) (PLLA), poly(d-lactic acid) (PDLA) and poly(l,d-lactic acid) (PLDLA) [79] . PLA is eco-friendly, biocompatible and tailorable polymer. Despite these advantageous properties of PLA, it has some drawbacks such as brittleness, hydrophobicity and slow degradation rate due to crystallinity and molecular weight [80]. Unlike PCL, PLA can be ingested by animals and humans through several enzymes such as proteinase K, pronase and bromelain. Besides, it can also be degraded through the mechanism of non-enzymatic hydrolysis like PCL [81].

Apart from these, processibility and tunability of PLA enables researchers to improve its mechanical characteristics in order to be used in bone tissue engineering and potential orthopedic applications. For this purpose, PLLA and PCL are blended to cover physical, thermal and mechanical inadequacies of each other. PCL is a flexible material with low stiffness. While PCL has elastic modulus about 0.21–0.44 GPa, PLLA has an elastic modulus approximately 4 GPa. High elastic modulus is associated with high stiffness; therefore PCL improves the mechanical properties of pure PLLA by increasing flexibility of it. PLLA has Tg range between 50-80 ℃, meaning that it is glassy at body temperature. Conversely PCL has a rubberlike structure ($Tg \cong -60^{\circ}C$) at body temperature, resulting in strengthened PLLA when they are blended. Furthermore, the degradation products of PLLA are known to decrease local pH and stimulate inflammation. Mixing PLLA with

PCL reduces acidification and diminishes the inflammatory response [82]. Additionally, both PCL and PLLA are FDA approved biomaterials, which can be used in *in vivo* bone tissue engineering applications without causing any detriment.

Polyurethanes (PUR) contain polymers that demonstrate linear, branched and cross-linked architectures. This structural diversity allows polyurethanes to be built either by homopolymers or copolymers including polyester or polyether sections, which are able to be used in wide variety of applications [83]. The basic structure of polyurethane consist of repeating urethane fragments derived from carbamic acids [84]. Moreover, polyurethanes are tough, biocompatible and stable polymers which can be suitable for bone tissue engineering and its medical applications [85].

Polyanhydrides are widely studied class of biopolymers due to their biodegradability and biocompatibility. They are generally synthesized by the method of melt condensation applied to diacids/diacidic esters or dehydration of the diacids. Additionally, they can also be synthesized via ROP of anhydrides and interfacial condensation. High molecular weight polyanhydrides are produced with melt condensation.

Polyanhydrides are degraded by surface erosion. The anhydride linkage is cleaved via hydrolysis and the rate of hydrolytic degradation varies according to the structure of polymer backbone. Although polyanhydrides are ideal polymers for drug delivery applications due to their surface eroding properties, they are mechanically weak materials accompanied with limited load bearing capacity which makes them unsuitable for orthopaedic applications. Therefore, to overcome this drawback poly(anhydride-*co*-imides),including imides that strengthens the polymer backbones, have been developed.

The most extensively examined polyanhydride is poly[(carboxyphenoxy propane)-(sebacic acid)] (PCPP-SA). It is a crystalline structure polymer which degrades rapidly and the degradation products are non-toxic and biocompatible. This polymer has FDA approval to be used as a controlled and localized drug delivery vehicle [86, 87].

Poly(propylene fumarate) (PPF), poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)), and oligo(poly(ethylene glycol) fumarate) (OPF) are the examples of fumarate based

polymers. Each of these materials has different physical and chemical properties, all of which can be customized according to the tissue to be studied. PPF is the most investigated biopolymer within those three fumarate based polymers. It is a linear polymer that has repeating units containing two ester bonds and one unsaturated carbon–carbon double bond. The degradation of PPF occurs via hydrolysis of the ester bonds. Fumaric acid (which is involved in Krebs cycle) and propylene glycol are the two degradation products of PPF. Furthermore, PPF can be cross-linked through its double bonds (unsaturated sites of PPF) to become biocompatible, biodegradable and mechanically strong polymeric networks [87, 88].

2.4. GROWTH FACTORS RELATED TO OSTEOGENESIS

Growth factors are signaling molecules that regulates cell functions by binding to their specific transmembrane receptors on target cells. Upon binding to their specific target receptors, they trigger an intracellular signal-transduction cascade that eventually reaches the nucleus of the cells and initiates the synthesis of associated proteins [89, 90]. Those proteins might have chemotactic (direction of cell migration), mitogenic (trigger of cell division), morphogenic (stimulation of cellular differentiation), apoptotic (initiation of cell death) and metabolic (arrangement of metabolic activity) effects on cells [91].

Insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and proteins from the transforming growth factor beta (TGF-β) superfamily (bone morphogenetic proteins (BMPs)) are the growth factors included in the biological activities of bone and other connective tissues [92].

In 1965, BMPs were discovered by Urist in bovine bone as a material that stimulated the ectopic bone formation in rats [93]. BMPs are molecules that have the ability of stimulating new bone formation. They are members of (TGF-β) superfamily, which possesses 15 distinct types of BMP (BMP 1 to BMP 15). BMP-2, BMP-4, BMP-6, BMP-7, and BMP-9 are capable of inducing osteogenic differentiation. BMP-2 is widely studied growth factor among the others, and it stimulates osteogenic differentiation of several cell types including osteoprogenitor cells, adipose derived mesenchymal stem cells, bone

marrow mesenchymal stem cells, muscle-derived stem cells, fibroblasts and chondrocytes. BMP-2 can be produced by human recombinant technology (rhBMP-2) in large amounts without losing its original bone induction capacity [94, 95]. It is proven that, rhBMP-2 (25–400 ng/ mL) is able to up-regulate genes correlated with osteogenic differentiation and down-regulate genes associated with myogenic differentiation [96]. In addition, osteogenic differentiation is regulated via Smad1/5/8 and MAPK downstream signaling pathway activated by BMP 2, 4, 6, 7 and 9 [92].

The IGF family includes two members, IGF-I and IGF-II. While IGF-II is thought to needed for embryonic development, IGF-I is indispensable later in life [97]. The IGFs are mitogenic peptides, which induce synthesis of DNA, promoting cell cycle progression and cell proliferation [98]. They are also involved in osteoblastic cell proliferation and bone matrix formation [99].

FGFs belong to a family consisting of 13 polypeptides. FGFs and their receptors (FGFRs) regulate the embryonic and postnatal skeletal development. They regulate osteoblastogenesis by either activating the Ras-dependent pathway causing mitogenesis stimulation, or triggering the STAT signaling pathways resulting in inhibition of mitogenesis [100, 101].

In 1970s, PDGFs were discovered as a platelet-dependent serum element, which has mitogenic effect on the fibroblasts, arterial smooth muscle cells, and glial cells. PDGFs are crucial in the regulation of embryonic development, particularly in the formation of vessels and organs. Among two receptors of PDGFs (PDGFRα and PDGFRβ), PDGFRα controls the signaling of gastrulation and the development of several organs such as lung, bones, intestine, testis, kidney, skin and neuroprotective tissue, whereas PDGFRβ regulates the formation of blood cellular components and blood vessels [102].

EGFs are proteins which have a major role in the mechanism of bone-remodeling regulation along with their specific receptors (epidermal growth factor receptors (EGFRs)). EGF together with EGFR initiates a signaling cascade to induce proliferation of mesenchymal osteoprogenitor cells (pre-osteoblasts and pre-chondrocytes). On the other

hand, it impedes the terminal differentiation of osteoprogenitor cells to mature osteoblasts and chondrocytes [103].

2.5. CELL TYPES

Cells have crucial importance in tissue engineering. They produce ECM elements and synthesize proteins depending on the specific tissues. In order to be used in tissue engineering applications, cells should fulfill certain requirements such as expandability, availability in adequate amounts, compatibility (abstaining from immune response) and capability to survive and maintain its function [104].

Cells can be isolated from autologous, allogeneic or xenogeneic sources for bone tissue engineering applications. Autologous cells are harvested directly from the patient's own body, therefore decreasing the risk of antigenicity. They do not have disease transmission risk but they possess risk of transfection and donor site morbidity due to invasive cell harvesting process. Allograft cells are isolated from human donors and transferred into another human patient. The allograft cell sources can either be obtained from living donors or cadaveric tissue. However, allografts have the probability of graft rejection and disease transmission, which create big problems in terms of tissue engineering practices. Xenogenic cells are obtained from different species such as pigs and baboons. Although xenogenic cells are utilized in the treatment of several disorders such as Parkinson’s disease, Huntington`s disease, diabetes or in heart valve replacement, they have the potential risk of disease transmission from animals to humans [104, 105].

Bone tissue specific cells, osteoblasts, osteoclasts and osteocytes can be used in bone tissue engineering. However, stem cells are widely investigated as cell types for bone tissue engineering applications nowadays. Stem cells are classified in three groups as follows: (1) embryonic stem cells (ESCs), (2) adult stem cells, and (3) induced pluripotent stem cells (iPS cells).Adult stem cells are extensively used in bone tissue engineering. Mesenchymal stem cells are adult stem cells which can differentiate into muscle, bone, cartilage and adipose tissue. They were discovered in 1966 by Friedenstein and his co-workers who harvested bone and cartilage progenitor cells from rat bone marrow stem cells. MSCs present in the structure of many organs and connective tissues, consisting of bone marrow,

gut, lung, liver, adipose, dental pulp, periodontal ligament and umbilical cord blood, all of which can be used as a source of MSCs. High proliferative capacity, easy isolation, multipotency and low immunogenicity make MSCs an excellent stem cell source for cell therapies [104, 105, 39].

Human adipose derived mesenchymal stem cells (hADMSCs) are isolated from adipose tissue via needle biopsy or lipoaspiration with minimal donor site morbidity. Since adipose tissue is used as a stem cell source, large amount of adult stem cells can be harvested compared to other mesenchymal stem cell resources. It was found that, while adipose tissue contains 1 stem cell in every 100 mononuclear cell, bone marrow originated mononuclear cells have 1 stem cell in 100,000. Therefore, hADMSCs are more preferable than human bone marrow mesenchymal stem cells in terms of accessibility and availability [106].

hADMSCs express the cell surface markers CD 29, CD 44, CD 71, CD 90, CD105, and SH3 but do not express STRO-1 and the hematopoietic markers CD 34 and CD 45. These markers are important for characterization of those stem cells [107].

Moreover, hADMSCs can differentiate into neurons, bone cells, cartilage cells, adipose tissue cells, endothelial and smooth muscle cells. Additionally, they possess some regenerative properties including secretion of growth factors, cytokines, proteases etc. and immunomodulation. Immunomodulation is initiated with the synthesis and secretion of immunosuppressive factors such as Interleukin-10 (IL10)/transforming growth factor-beta (TGF-β) and galectin 1 to promote maturation of suppressor T cell and induce T cell death respectively [108]. All of these properties showed that hADMSCs have a great potential in regenerative medicine and bone tissue engineering applications.

2.6. OBJECTIVES OF THE STUDY

In this study, the effect of BMP-2 loaded microsphere embedded double layer PCL-PLLA nanofibrous scaffold system on osteogenic differentiation capacity of human adipose derived mesenchymal stem cells (hADMSCs) was investigated. For this purpose, the scaffolds were prepared with electrospinning method and hADMSCs were seeded on them. Osteoinductive capacity and the biocompatibility of the polymers were examined via certain experimental methods.

3. MATERIALS

3.1. SCAFFOLD PREPARATION

- PLLA (Mw: 101,700, prod. no.93578) (Sigma-Aldrich, USA)
- PCL (Mn: 70,000 – 90,000, pro. no.440744) (Sigma-Aldrich, USA)
- Gelatin (type B, from bovine skin, prod. no. 48723) (Fluka, Switzerland)
- Chloroform (Riedel-de Haen, Germany)
- 2,2,2 – trifluoroethanol (TFE) (Riedel-de Haen,Germany)
- Methanol (Riedel-de Haen, Germany)
- Gluteraldehyde Solution (GA, 50%. prod. no. G7651) (Sigma-Aldrich, USA)
- Acetone (Merck, Germany)
- Bone Morphogenetic Protein-2 (BMP-2) (human recombinant, prod. no. H4791-10UG), (Sigma-Aldrich, USA)
- High Voltage Power Supply (Gamma High Voltage, USA)
- Syringe Pump (NewEra Syringe Pump System, USA)

3.2. CHARACTERIZATION OF POLYMER STRUCTURE BY SCANNING ELECTRON MICROSCOPY

- Cacodylic Acid Sodium Salt Trihydrate (AppliChem, Germany)
- Glutaraldehyde Solution, Grade I, 25% (Sigma-Aldrich, Germany)
- Sputter Coater (Bal-tec SCD 005, Germany)
- Scanning Electron Microscope (Carl Zeiss EVO, Germany)

3.3. *IN VITRO* CELL CULTURE STUDIES

3.3.1. Isolation of Human Adipose Derived Mesenchymal Stem Cells (hADMSCs)

- Collagenase Solution:
  - Bovine Serum Albumin (BSA) (Sigma-Aldrich, USA)
  - Collagenase I (Life Technologies, USA)
  - Phosphate Buffered Saline (DPBS, 1X) (Gibco–Life Technologies, USA)
  - HEPES (Sigma-Aldrich, USA)

- Erythrocyte Lysis Buffer:
  - Ammonium Chloride (Sigma-Aldrich, USA)
  - Potassium Hydrogen Carbonate (Sigma-Aldrich, USA)
  - Ethylenediaminetetraacetic Acid (EDTA) (Merck, Germany)

- Phosphate Buffered Saline (DPBS, 1X) (Gibco–Life Technologies, USA)
- Dulbecco's Modified Eagle Medium / F12 (1:1), (Ham)1X (Gibco – Invitrogen, USA)
- Human Recombinant Fibroblast Growth Factor -2 (FGF-2) (ref. no. SRP4037-50 UG) (Sigma-Aldrich, USA)
- Penicillin-Streptomycin (Gibco – Life Technologies, USA)
- Fetal Bovine Serum (Gibco-Invitrogen, USA)
- T150 Tissue Culture Flask (TPP, Switzerland)
- Centrifuge (Santrifuj Hettich-Rotofix32A, Germany)
- Laminer Cabinet (Telstar, Bio-II-A, Spain)
- Inverted Microscope (Nikon Eclipse TC 100, USA)

3.3.2. Characterization of hADMSCs

- Mouse Anti-Human CD90 Antibody (BD Biosciences, USA)
- Rat Anti-Human CD44 Antibody (Abcam, USA)
- Mouse Anti-Human CD24 Antibody (BD Biosciences, USA)

- Mouse Anti-Human CD 117 –(C-Kit) Antibody (eBioscience, USA)
- Phosphate Buffered Saline (D-PBS, 500 mM, pH 7.4) (Gibco - Invitrogen, USA)
- FACS Calibur (BectonDickenson, USA)
- Round Bottom Test Tubes

3.3.3. Cell Seeding on PCL-PLLA Single/Double Layer Scaffolds

- hADMSCs
- PCL-PLLA Single/Double Layer Scaffolds
- Dulbecco's Modified Eagle Medium / F12 (1:1), (Ham)1X (Gibco – Invitrogen, USA)
- Human Recombinant Fibroblast Growth Factor -2 (FGF-2) (ref. no. SRP4037-50 UG) (Sigma-Aldrich)
- Penicillin-Streptomycin (Gibco – Life Technologies, USA)
- Fetal Bovine Serum (Gibco-Invitrogen, USA)
- Phosphate Buffered Saline (DPBS, 1X) (Gibco–Life Technologies, USA)
- Trypsin-EDTA (1X, 0.25%) (Gibco – Invitrogen, USA)
- T 150 Tissue Culture Flask (TPP-Switzerland)
- 24 – well Cell Culture Plate (Corning)
- Hemocytometer (Hausser Bright-Line, USA)
- Inverted Microscope (Nikon Eclipse TC 100, USA)
- Centrifuge (Santrifuj Hettich-Rotofix32A, Germany)
- Laminar Cabinet (Telstar, Bio-II-A, Spain)
- Ultra Violet (UV) Light

3.3.4. Differentiation of hADMSCs

- hADMSCs seeded on PCL-PLLA Single/Double Layer Scaffolds
- Dulbecco's Modified Eagle Medium / F12 (1:1), (Ham) 1X (Gibco – Invitrogen, USA)
- Penicillin-Streptomycin (Gibco – Life Technologies, USA)
- Fetal Bovine Serum (Gibco-Invitrogen, USA)

- Dexamethasone (AppliChem, Germany)
- β- Glycerophosphate (AppliChem, Germany)
- Ascorbic Acid (AppliChem, Germany)

3.4. CHARACTERIZATION OF CELL DIFFERENTIATION

3.4.1. Cell Viability Assay

- CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA)
- Dulbecco's Modified Eagle Medium (DMEM - 1g/L glucose) (Gibco - Invitrogen, USA)
- 96-well Cell Culture Plates (Orange Scientific, Belgium)
- Elisa Plate Reader (Bio-Tek, El x 800, USA)

3.4.2. Von Kossa Staining

- Von Kossa Staining Kit (Diagnostic Biosystems, USA)
- Phosphate Buffered Saline (DPBS, 1X) (Gibco–Life Technologies, USA)
- Stereo Microscope (KL 1500, Leica, Germany)

3.4.3. Alkaline Phosphatase (ALP) Assay

- ALP Assay Kit (Randox Laboratories, UK)
- Ultrasonic Homogenizer
- Centrifuge (Eppendorf 5810 R, Germany)
- Tris Buffer (0.1 M, pH:9)
- Triton™ X-100 (Sigma-Aldrich, USA)
- Phosphate Buffered Saline (DPBS, 1X) (Gibco–Life Technologies, USA)
- Falcon Tubes
- 96-well Cell Culture Plates (Orange Scientific, Belgium)
- Elisa Plate Reader (Bio-Tek, El x 800, USA)

3.4.4. Total Protein Quantification

- Tris Buffer (0.1 M, pH:9)
- Triton™ X-100 (Sigma-Aldrich, USA)
- Ultrasonic Homogenizer
- Smart™ micro BCA Protein Assay Kit (Intron Biotechnology, Korea)
- 96-well Cell Culture Plates (Orange Scientific, Belgium)
- Elisa Plate Reader (Bio-Tek, El x 800, USA)

3.4.5. Confocal Microscopy Analysis

- Collagen Type I Mouse Monoclonal Antibody (Santa Cruz Biotechnology, USA)
- Osteocalcin Mouse Monoclonal Antibody (Santa Cruz Biotechnology, USA)
- 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, USA)
- Alexa Fluor® 488 Goat Anti-mouse IgG Antibody (Life Technologies, USA)
- Formaldehyde (Fluka, Switzerland)
- Tween® 20 (AppliChem, Germany)
- Phosphate Buffered Saline (DPBS, 1X) (Gibco–Life Technologies, USA)
- Fetal Bovine Serum (Gibco-Invitrogen, USA)
- Microscope slides and coverslips
- Prolong Gold-Antifade Reagent (Invitrogen, USA)
- Confocal Microscope (Carl Zeiss, Germany)

4. METHODS

4.1. PREPARATION OF PCL-PLLA SINGLE/DOUBLE LAYER SCAFFOLDS

The scaffolds were fabricated with respect to the procedure developed by *Gungor Ozkerim et al.* [109]. Briefly, PCL-PLLA solutions (10 % w/v) were dissolved in a mixture of chloroform-methanol (3:1 v/v) with an equal ratio (1:1 w/w). Then the polymer solutions were stirred at least 5 h at room temperature. Subsequently, the solutions were placed into the syringe pump system and 20 kV voltage was applied for the electrospinning process. The flow rate of the solutions was 3 mL/h and the tip-to-collector distance was 15 cm. Both single layer and the top and the bottom layers of double layer PCL-PLLA scaffolds were prepared according to these parameters.

For double layer scaffolds, BMP-2 loaded microspheres were prepared in accordance with the method described by [109]. BMP-2 was dissolved in PBS (0,01 M, pH 7.4) and then mixed with 15 wt. % aqueous gelatin solution. The mixture was poured into olive oil (40 ℃) and stirred at 500 rpm. The gluteraldehyde solution (7.5%) was added a few minutes later and the mixture was cooled down. After overnight incubation of the emulsion on the stirrer, resulting microspheres were gathered by centrifugation subsequent to washing step with acetone. Microspheres were left to dry at 4 ℃ for 24 h before use. For the integration of the whole system, PCL-PLLA nanofibers were collected from the electrospinning instrument and then BMP-2 loaded microspheres (suspended in 70 % ethanol solution) were spread on the fiber layer. For the construction of the upper layer, air-dried nanofibers (for evaporation of ethanol) were placed into electrospinning system again to cover it with the second layer of PCL-PLLA nanofibers.

4.2. CHARACTERIZATION OF POLYMER STRUCTURE BY SCANNING ELECTRON MICROSCOPY

The morphology of the cell-seeded and empty scaffolds was examined by scanning electron microscopy analysis. The scaffolds were washed with cacodylate buffer (0.1 M, pH 7.4) for three times then they were incubated in 2.5% (v/v) gluteraldehyde solution for 1 h for cell fixation (after 10 and 20 days of incubation). Subsequently, the scaffolds had mounted on metal discs and they were coated with gold (10 nm in thickness) particles by sputter coater. Finally, all of the scaffolds were examined via Carl Zeiss EVO Scanning Electron Microscope operated at an acceleration of 10.00 kV.

4.3. *IN VITRO* CELL CULTURE STUDIES

4.3.1. Isolation of Human Adipose Derived Mesenchymal Stem Cells (hADMSCs)

Adipose tissue was obtained from upper inner thighs of female patients undergoing liposuction procedure at Yeditepe University Hospital. Initially, the adipose tissue and collagenase solution were mixed at a ratio 1:1 and digested for 1h with continuous shaking at 170 rpm), at 37℃. The digested tissue was transferred into a 50 mL falcon tube and the rest of the tube was filled with the erythrocyte lysis buffer and then centrifuged at 2,500 rpm for 7 min at room temperature. The supernatant part of the solution was discarded and the pellet was resuspended with 2 mL of erythrocyte lysis buffer. The cell suspension was transferred into a new falcon tube and the volume was completed to 50 mL with erythrocyte lysis buffer. After 10 min. of incubation at 37 ℃ with continuous shaking at 170 rpm, the cell suspension was centrifuged at 1,400 rpm for 7 min at room temperature. The cells in the pellet were washed with PBS (1 X, without Ca/Mg) and subsequently centrifuged at 1,400 rpm for 7 min at room temperature. Cell pellet was resuspended in 6-10 mL expansion medium containing DMEM F-12 (1:1) (1X) Ham`s Nutrient mixture with 10 % FBS, 1 ng/mL FGF-2 and 1% penicillin streptomycin (100 U/mL Penicilium and 100 μg/mL Streptomycin). Finally, the cells were filtered through 100 μm cell strainer

and seeded on T 150 flasks. Cells were incubated in CO_2 incubator and medium was refreshed every other day.

4.3.2. Characterization of hADMSCs

Characterization of hADMSCs was carried out using flow cytometry. At the beginning, the cells were washed with PBS, trypsinized and centrifuged at 1,500 rpm for 5 min. The pellet was dissolved in medium and the cells were counted with hemocytometer. The cells were separated into tubes so that each tube possesses 300,000 cells. PBS (2 mL) was added into tubes and then, the tubes were centrifuged at 2,000 rpm for 5 min. The supernatant was discarded and the pellet was dissolved in 1 mL. After centrifugation at 2 000 rpm for 5 min, the supernatant was discarded and CD markers (CD 90, CD 44, CD 24, CD 117) were added on cells in the dark. Later, the cells were incubated for 45 min at room temperature. The cells were centrifuged at 2,000 rpm for 5 min for the last time and the pellet was dissolved in PBS. Finally, the cells were analyzed with flow cytometer instrument.

4.3.3. Cell Seeding on PCL-PLLA Single/Double Layer Scaffolds

At the beginning, PCL-PLLA single/double layer scaffolds were cut into the shape of a square (length-width ≅1cm-1cm). The scaffolds were placed into the wells of 24-well plates separately, and then, sterilized under UV-light for 1h. Meanwhile, cells were trypsinized and centrifuged at 1,500 rpm for 5 minutes. The cells were counted with hemocytometer under inverted microscope and then cells were seeded onto each scaffold (20,000 cells/scaffold). The cell-seeded scaffolds were incubated at 37 ℃ in CO_2 incubator for further experiments. Medium was refreshed twice a week.

4.3.4. Differentiation of hADMSCs

The differentiation medium was prepared to induce osteogenic differentiation of hADMSCs. The medium was composed of 0,1 μM dexamethasone, 10 m$M\beta$-glycerophosphate and 50 μg/ mL ascorbic acid. DMEM/F-12 (1:1) (Ham) 1X was used as basal medium. Besides, 1% penicillin streptomycin and 10% FBS were added into the

medium. Next day, the osteogenic differentiation medium was added on hADMSCs seeded scaffolds and replenished semiweekly.

4.4. CHARACTERIZATION OF CELL DIFFERENTIATION

4.4.1. Cell Viability Assay

CellTiter 96® AQueous One Solution Cell Proliferation Assay was used to determine the number of viable cells on PCL-PLLA single/double layer scaffolds. The assay was performed at days 1, 7, and 14 subsequent to cell seeding. For that purpose, DMEM - 1g/L glucose was mixed with MTS solution with a ratio 5:1. Meanwhile, the cell-seeded scaffolds were transferred into a sterile 24-well plate and washed with PBS for three times. DMEM-MTS mixture (500 μL) was added into each sample and then incubated for 3 h in CO_2 incubator at 37 ℃. Finally, 200 μL of the reacted solution from each well was transferred into a 96-well plate and the absorbance was measured at 490 nm with Elisa Plate Reader.

4.4.2. Von Kossa Staining

The Von Kossa kit was used to stain calcium deposits on cell-seeded scaffolds. Firstly, silver nitrate solution (5%) was added onto the scaffolds and they were subjected to UV light for 30 min. Then, the scaffolds were incubated in sodium thiosulfate solution (5%) for 5 min. The nuclear fast red solution was added onto samples and incubated for 5 min. The scaffolds were rinsed with PBS between each step. Eventually, the scaffolds were visualized under stereo microscope.

4.4.3. Alkaline Phosphatase (ALP) Assay

The assay was done to assess ALP activity in differentiated cells. At first, cell-seeded scaffolds were washed with PBS and then, they transferred into falcons containing 500 μL mixture of Tris buffer (0.1 M, pH 9.0) and 0.01 % Triton® X-100. The scaffolds were sonicated after they were exposed to freeze-thaw cycles. Following sonication, samples were centrifuged at 2,000 rpm for 10 min and the supernatant part (100 μL) was used for

the ALP assay. The p-nitrophenol phosphate (p-NPP) solution supplied by ALP kit and the sample were mixed with a ratio of 1:1. The absorbance values were recorded in every two minutes (between 0-16 minutes) using Elisa Plate Reader at 405 nm. The ALP activity levels of each sample were calculated from the slopes of the absorbance-time graphics. The determined activity levels were normalized by the total amount of protein obtained from the same samples. The total protein content was detected by using Micro BCA protein assay kit according to the manufacturer`s instructions.

4.4.4. Confocal Microscopy Analysis

Confocal microscopy analysis was performed to observe osteogenic markers, collagen type I and osteocalcin, at days 14, 24 and 20, 27 respectively. Cells on the electrospun scaffolds were fixed with 3.7 % (w/v) formaldehyde including 0.001 % (v/v) Tween® 20 for 30 min. Later, formaldehyde solution was discarded and the scaffolds were washed with PBS. Cell-seeded scaffolds were incubated in blocking solution (3 % FBS in PBS (v/v)) for 10 min to avoid non-specific binding of the fluorescent dyes.

The indirect immunofluorescence staining method was used to visualize osteogenic markers. Initially, collagen type I and osteocalcin primary antibodies were diluted with PBS solution containing 1.5 % FBS (at a ratio of 1:100) in separate tubes. The antibodies were added on cell-seeded scaffolds individually and incubated for 1 h at 37℃. Thereafter samples were rinsed with PBS and Alexafluor 488 conjugated secondary antibodies (diluted with a ratio of 1:100 in PBS solution containing 1.5 % FBS) were added onto samples. After 1h incubation at 37℃, the scaffolds were washed with PBS for three times. Following washing steps, the cells were stained with DAPI to visualize nuclei of the cells. DAPI was diluted with 1:100 ratio using PBS and the cell-seeded scaffolds were incubated in DAPI solution for 15 min at room temperature. The samples were rinsed with PBS for the last time and left for air-drying. The air-dried scaffolds were placed onto glass microscope slides, and then Prolong Gold Antifade Reagent was dropped on the scaffolds to prevent from photobleaching of the dye. Finally, the cell-seeded scaffolds were investigated under confocal microscope.

5. RESULTS

5.1. CHARACTERIZATION OF HUMAN ADIPOSE DERIVED MESENCHYMAL STEM CELLS

The human adipose tissue stem cells were characterized by fluorescence-activated cell sorter (FACS) using FITC, PE or APC conjugated antibodies. The specific cell surface markers; CD 90, CD44, CD 24 and CD 117 were used to determine mesenchymal stem cells (MSC). CD 90 (glycophosphatidylinositol anchor protein) and CD 44 (glycoprotein) antigens are the cell surface proteins expressed by mesenchymal stem cells. CD 24 and CD 117 are the hematopoietic stem cells markers and they are not expressed by mesenchymal stem cells.

According to the Table 5.1.and Figure 5.1, hADMSCs gave positive results for CD 90 (96.7 %) and CD 44 (92.9 %). On the contrary, hADMSCs were negative for CD 24 (1.23 %) and CD 117 (0.97 %) as it was expected.

Table 5.1.The FACS results of hADMSCs at passage 3

Cell Surface Antigens		**Histogram Results**
hADMSCs (Only Cells)		1.02 %
Positive Surface Markers for hADMSCs	CD 90	96.7 %
	CD 44	92.9 %
Negative Surface Markers for hADMSCs	CD 24	1.23 %
	CD 117	0.97 %

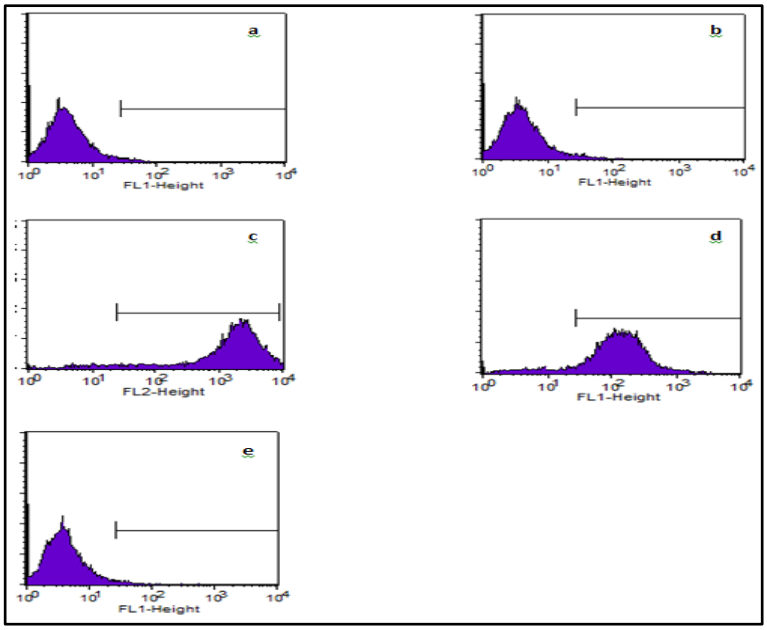

Figure 5.1. Flow cytometry histogram of cells isolated from human adipose tissue labelled with fluorescein conjugated antibodies; (a) Only cells without antibody, (b) CD 24, (c) CD 90, (d) CD 44, (e) CD 117

5.2. CHARACTERIZATION OF POLYMER STRUCTURE BY SCANNING ELECTRON MICROSCOPY

Scanning electron microscopy (SEM) analysis was performed to examine surface morphology, structure and architecture of the scaffolds. SEM utilizes a focused beam composed of high-energy electrons to produce topographic images of samples.

Figure 5.2 and Figure 5.3 demonstrate nanofibrous structures of PCL-PLLA single/double layer scaffolds. The fiber diameter of the PCL-PLLA scaffolds was measured as $\sim$ 1000 nm. The overall thickness of the PCL-PLLA double layer scaffold system was 289 ± 14 μm [109].

According to the SEM images of the hADMSC seeded PCL-PLLA double layer system (Fig 5.2 a and b), the cells attached, spread and produce extracellular matrix on nanofibrous scaffolds. The attachment of hADMSC on single layer PCL-PLLA scaffolds was also observed in Figures 5.3 a and b. The existence of cells was also supported with the SEM images of empty scaffolds (Figure 5.2 c, Figure 5.3 c).

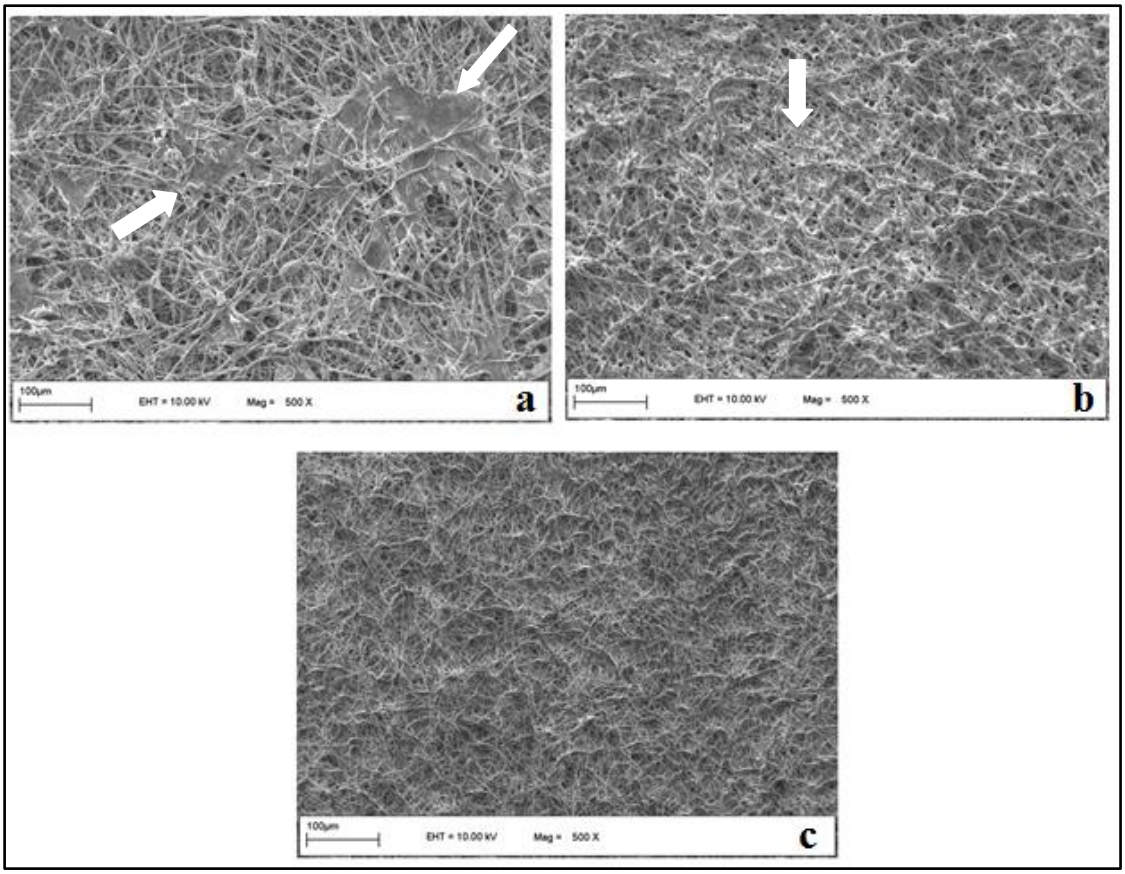

Figure 5.2. Scanning Electron Microscopy images of double layer PCL-PLLA scaffolds with 500 X magnification (a) after 10 days of incubation, (b) after 20 days of incubation, (c) empty scaffold (Arrowheads show presence of cells)

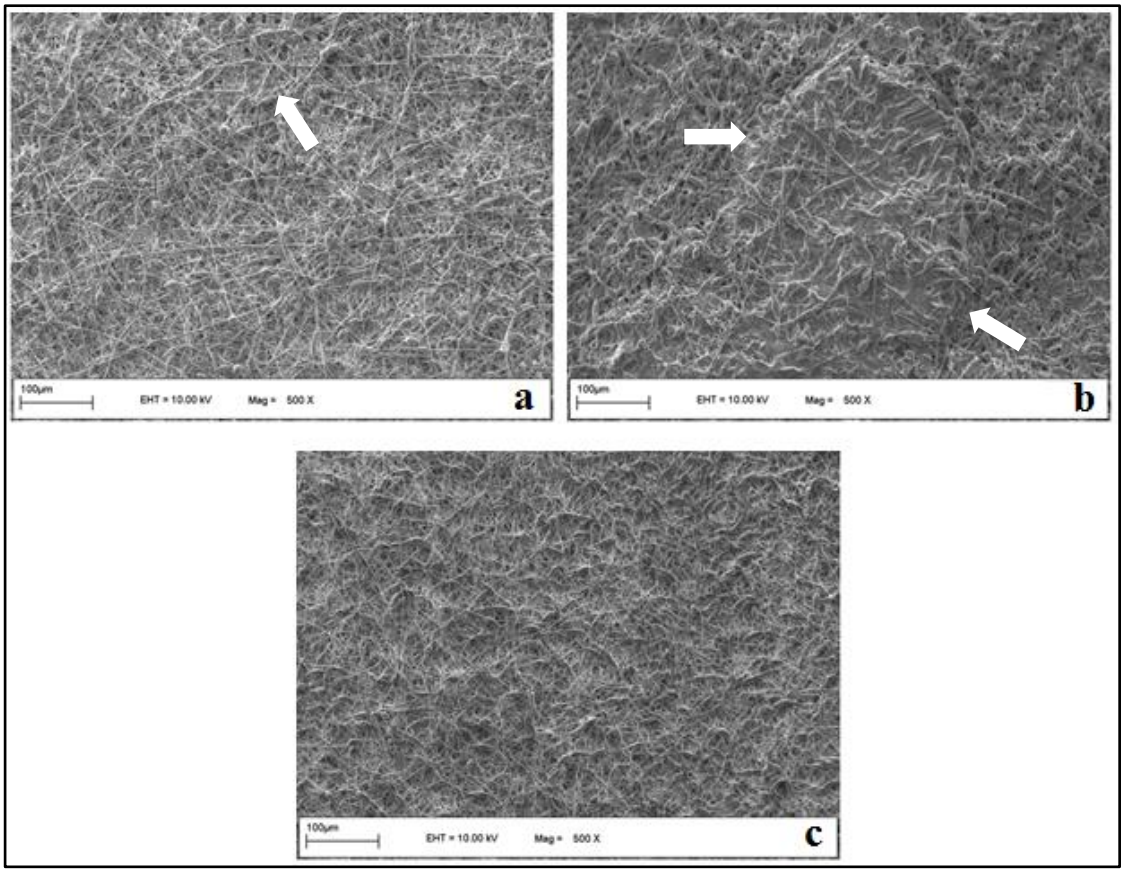

Figure 5.3. Scanning Electron Microscopy images of single layer PCL-PLLA scaffolds with 500 X magnification (a) after 10 days of incubation, (b) after 20 days of incubation, (c) empty scaffold (Arrowheads show presence of cells)

5.3. CELL VIABILITY ON PCL-PLLA SCAFFOLDS

The MTS assay was performed on days 1, 7 and 14 in order to ascertain hADMSCs` viability and proliferation on PCL-PLLA double layer and single layer scaffolds. The absorbance values of the cells seeded on PCL-PLLA double layer scaffolds were increased through day 1 to 14, in contrast a decrease in the absorbance values of the cells seeded on single layer PCL-PLLA scaffolds was observed (Figure 5.4).Additionally, hADMSCs (only cell) were seeded in the polystyrene 24 well plates (20,000 cells/well) to use them as positive control in the experiment.

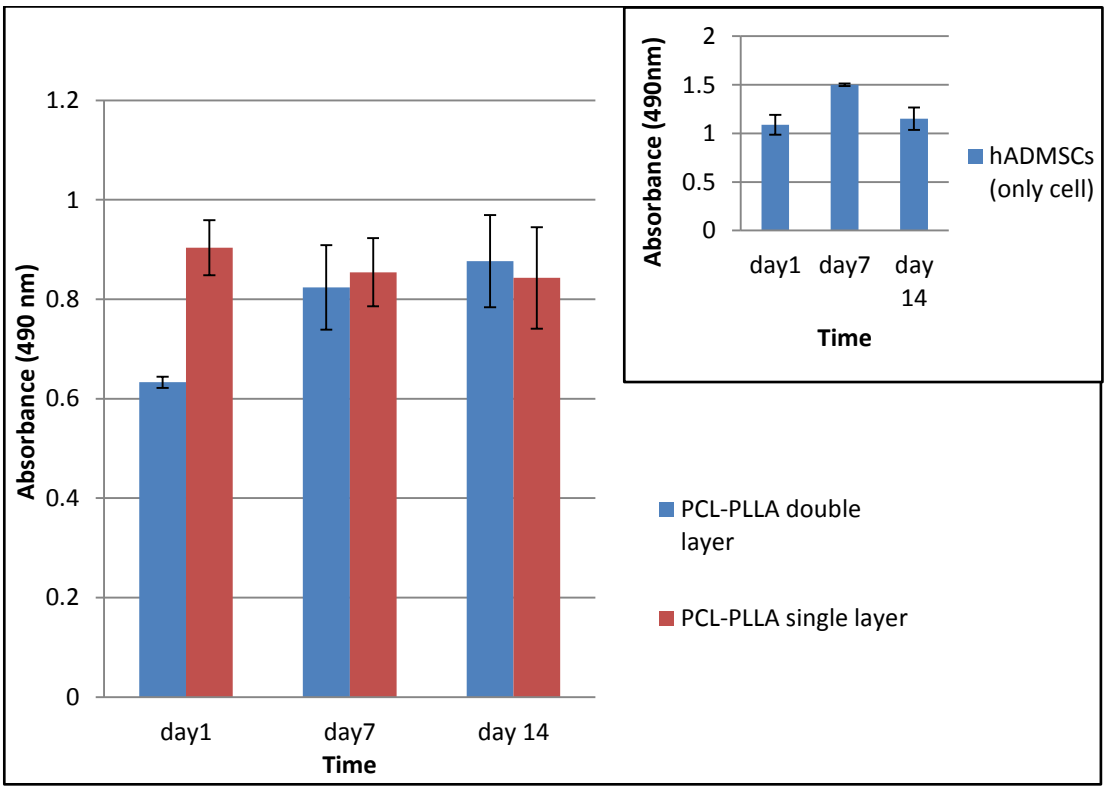

Figure 5.4. MTS cell viability assay of hADMSCs seeded PCL-PLLA single/double layer scaffolds on after 1, 7 and 14 days of incubation

A calibration curve was plotted to convert absorbance values into cell numbers. According to the calibration curve, the number of cells attached on the PCL-PLLA single/double layer scaffolds was calculated and a second column graph (cell number vs. time) was plotted (Figure 5.5). An increase in the number of the cells on double layer PCL-PLLA from day 1

to 14 was observed pursuant to the Figure 5.3, which indicates that hADMSCs were attached and proliferated on the PCL-PLLA double layer scaffolds throughout 14 days of incubation period.

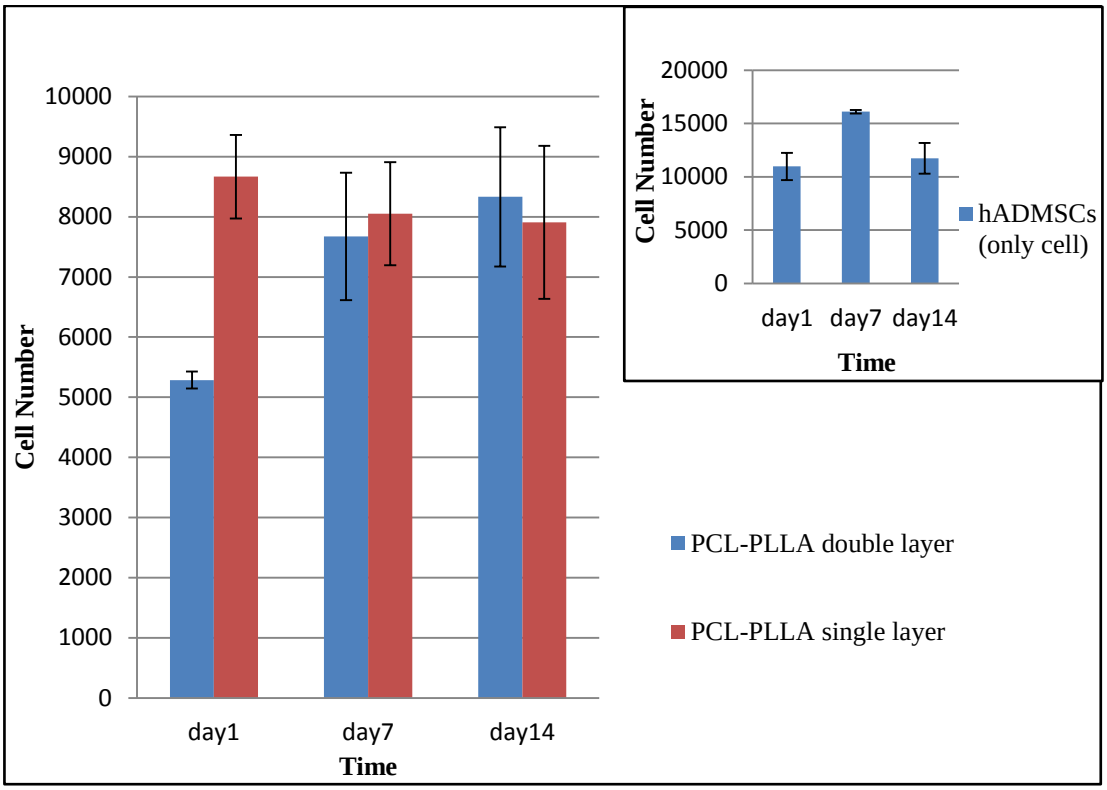

Figure 5.5. Cell proliferation assay of hADMSCs seeded PCL-PLLA single/double layer scaffolds after 1, 7 and 14 days of incubation (Initial cell seeding = 20,000 cells/well)

Although the initial cell attachment on single layer PCL-PLLA scaffolds (8,667 cells) was higher than that of double layer PCL-PLLA scaffolds (5286 cells).The number of the attached cells on double layer PCL-PLLA scaffolds (8,332 cells) was quite close to the number of cells attached on single layer scaffolds (7,908 cells) after 14 days of incubation. On the other hand, the number of hADMSCs seeded on polystyrene well plates (control) were increased through day 1 to 14 and then started to decrease since there was no place left for cells to attach and grow properly.

5.4. ALKALINE PHOSPHATASE ASSAY

Alkaline phosphatase (ALP) is an enzyme that catalyzes the formation of p-nitrophenylate in the presence of p-nitrophenylphosphate (pNPP) by removing phosphate from pNPP. As a result of this reaction, p-nitrophenylate gives yellow color to the solution and the intensity of the color is detected by Elisa Plate Reader at 405 nm.

ALP is highly expressed by osteoblasts for new bone formation and growth; therefore it is used as an early marker for osteogenic activity. According to Figure 5.6, ALP activity of the cells seeded on PCL-PLLA double layer scaffolds was decreased from day 1 to day 7 and reached its peak at day 14. From day 1 to day 14, ALP activity levels of the cells seeded on single layer PCL-PLLA scaffolds were increased. hADMSCs seeded on well-plates were used as a positive control group in the experimental procedure.

In order to comprehend the absolute quantity of the ALP enzyme produced by the hADMSCs, ALP activity was normalized to total protein content of cells. Figure 5.7 shows the normalized ALP levels of the cells. The levels of ALP were changed for the cells seeded on double/single layer PCL-PLLA scaffolds when compared to the ones in Figure 5.6. ALP expression levels of hADMSCs seeded on double layer PCL-PLLA scaffolds were increased through 14 days. At the end of day 14, ALP levels were increased 3 folds for double layer scaffolds. A sharp increase (~20 fold) in ALP levels was observed from day 1 to day 7 for PCL-PLLA single layer samples and at 14^{th} day ALP level was decreased 1.8 fold. After 14 days of incubation, the cells on double layer scaffolds expressed slightly more ALP than the cells on single layer.

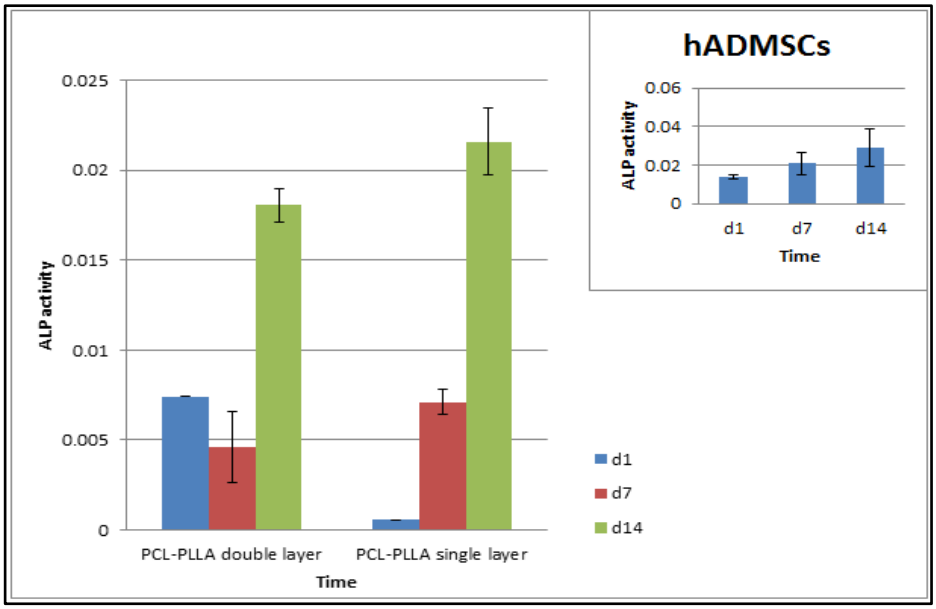

Figure 5.6.ALP activity of hAMSCs seeded on PCL-PLLA single/double layer scaffolds

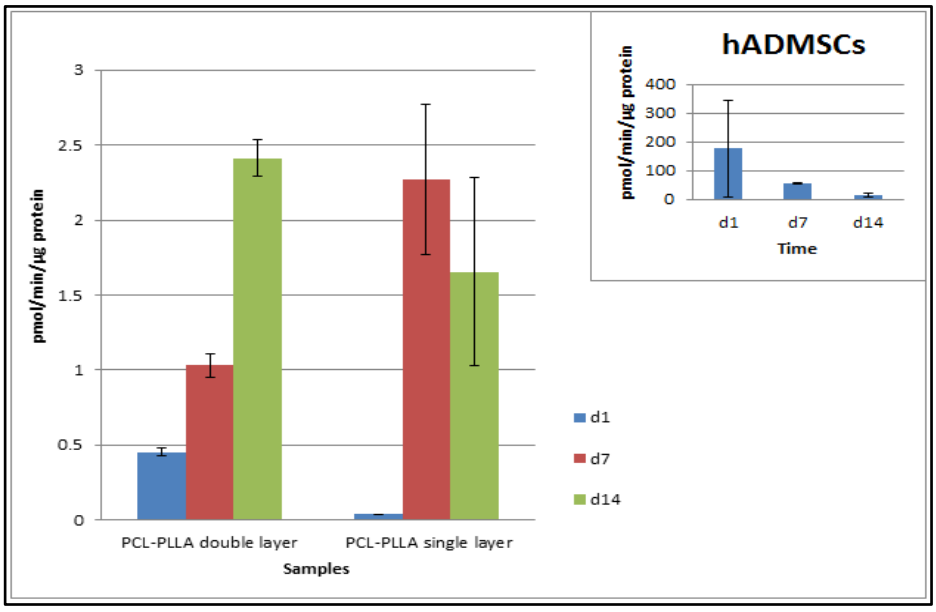

Figure 5.7.ALP activity normalized to protein concentration

5.5. VON-KOSSA STAINING

Von-Kossa staining method was applied to detect calcium deposits visually. In this method, silver nitrate solution was used to substitute carbonate or phosphate ions for silver ions for producing silver salts. The silver salts were exposed to UV light for reduction and then sodium thiosulfate was used to discard unreduced silver. Additionally, Nuclear Fast Red solution was used as a counterstain in this procedure.

The Figure 5.8 demonstrates brown calcium depositions on single/double layer PCL-PLLA scaffolds. The intensity of the color brown is directly proportional with the amount of mineralization.

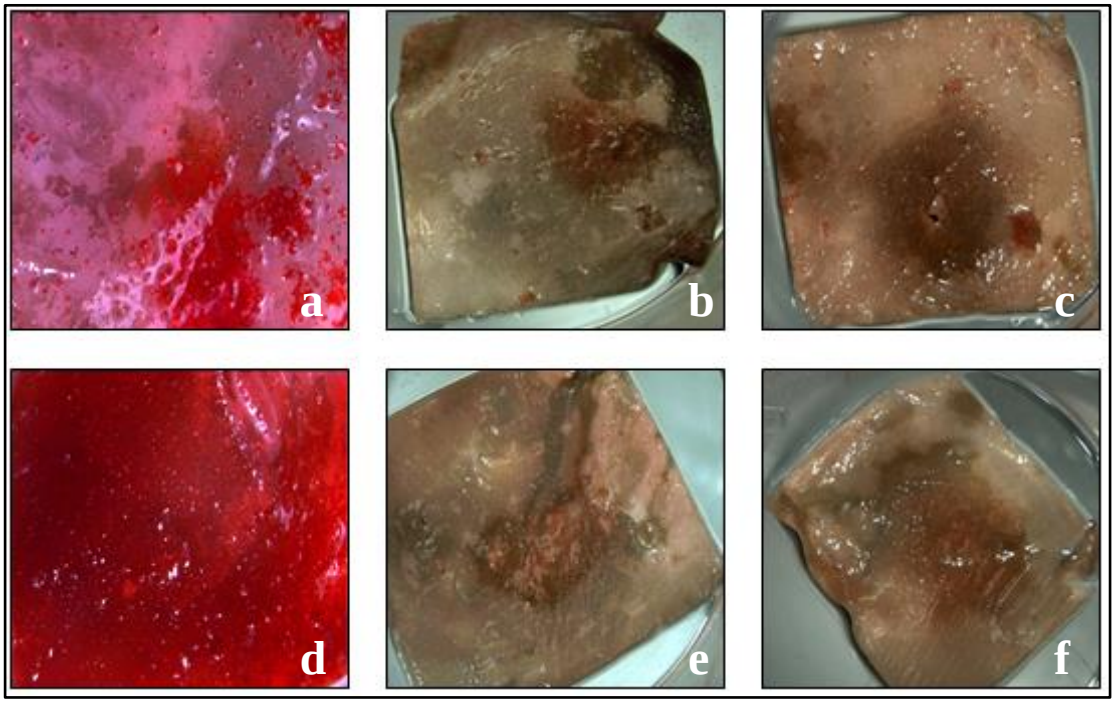

Figure 5.8. Calcium deposits visualized by Von Kossa Staining Method after (a, d) 7, (b, e) 14 and (c, f) 24 days of incubation on double layer PCL-PLLA scaffolds (a, b, c) and single layer PCL-PLLA scaffolds (d, e, f).

According to the Figure 5.8 a, d, the both types of the scaffold did not possess any calcium deposits at day 7, since they were only stained with Nuclear Fast Red dye. Qualitatively, the brown color of the calcium deposits on double layer PCL-PLLA scaffolds at days 14 and 24 (Figure 5.8 b, c) was darker than the brown color of the calcium deposits on single layer PCL-PLLA scaffolds at days 14 and 24 (Figure 5.8 e, f). Besides, after 24 days of incubation, it was observed that the double layer PCL-PLLA scaffolds had the darkest brown color of calcium deposits on them compared to the other samples. It means that the highest mineralization occurred on the double layer PCL-PLLA scaffolds.

Apart from these, the mineralization was observed on hADMSCs seeded well plates under light microscope after 14 and 24 days of incubation periods (Figure 5.9 b, c).

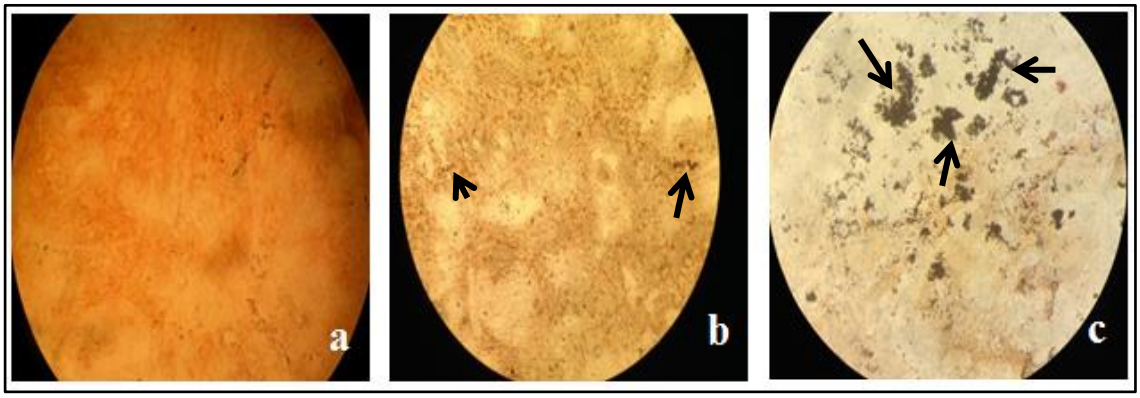

Figure 5.9. Von-Kossa staining of hADMSCs seeded on well-plates under the light microscope with 10 X objective (a) Day 7, (b) Day 14, (c) Day 24 (Arrowheads show the calcium deposits)

The ColorPic software tool was utilized to verify and support all of the qualitative observations about the lightness or darkness of the brown color of calcium deposits. The software determines the hue, saturation and value of the colors. It also demonstrates the value of primary colors red, green and blue. The hue of a color is described as the dominant wavelength of light that is emitted or reflected from an object. Saturation designates the purity of a color. The value is referred as the lightness of a color meaning that, the lower the value of a color, the darkest the color is. In light of this information, the darkest brown color was on the PCL-PLLA double layer scaffold at day 24 (value = 67) and the lightest brown color was on the single layer PCL-PLLA scaffold at day 14 (value =

96). According to this color values, mineralization of the cells on the scaffolds in Table 5.2 can be ranked as e < f < b < c.

Table 5.2. The data obtained from ColorPic (Iconico Inc.) tool with respect to the color intensity of calcium deposit images

		Color	Hue	Saturation	Value	Red	Green	Blue
PCL-PLLA Double Layer	DAY 14		24	196	**82**	82	44	19
	DAY 24		23	232	**67**	67	29	6
PCL-PLLA Single Layer	DAY 14		28	178	**96**	96	60	29
	DAY 24		25	181	**90**	90	53	26

5.6. CONFOCAL MICROSCOPY ANALYSIS

Confocal microscopy analysis was performed to visualize immunocytochemicallystained collagen type I and osteocalcin extracellular matrix elements on PCL-PLLA single/double layer scaffolds. Collagen type I is a glycoprotein produced by osteoblasts to generate collagen matrix in bone tissue. Osteocalcin is the most plentiful non-collagenous protein exists in the bone and it is also produced by osteoblasts. These two proteins were used as an osteogenic marker in this procedure.

Collagen type I and osteocalcin proteins were detected with their specific primary monoclonal antibodies. Alexa Fluor® 488 Goat Anti-mouse IgG Antibody was used as secondary antibody that binds to primary antibody. The secondary antibody is conjugated with a photostable fluorescent dye which gives green color when it is excited at 488 nm, meaning that both collagen type I and osteocalcin were stained with green fluorescent dye. DAPI is a blue fluorescent dye that binds A-T regions of DNA to make nuclei of the cell visible. In this method it was used for determination of cell localization.

Figure 5.10 demonstrates the collagen type I molecule expressed by the cells seeded on PCL-PLLA double layer scaffolds after 14 and 24 days of incubation. It can be observed that the intensity of green dye at day 24 (Figure 5.10 b) was higher than the intensity of the green dye at day 14 (Figure 5.10 a). From this comparison, it can be deduced that the expression of collagen type I molecules at day 24 on double layer scaffolds was higher than that of at day 14.

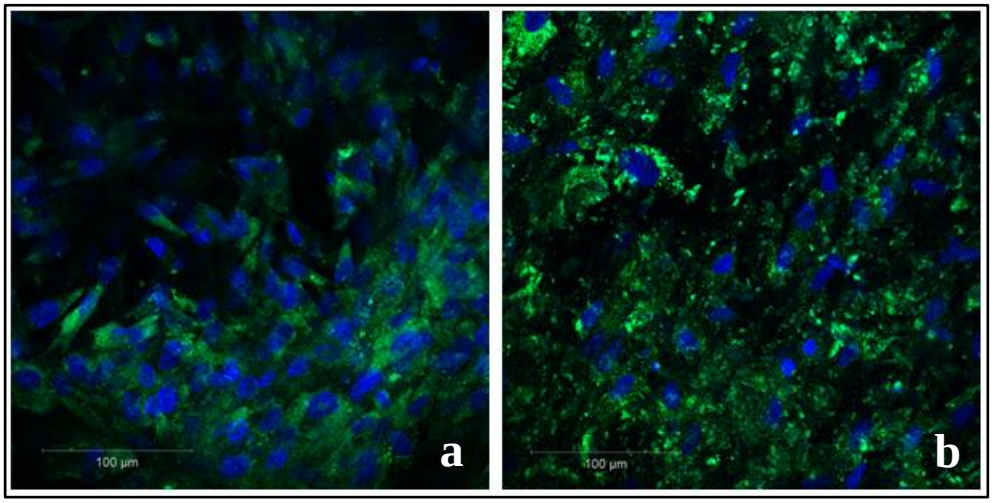

Figure 5.10. Confocal microscopy images of cells seeded on double layer PCL-PLLA scaffolds after (a) 14, (b) 24 days of incubation (with 20 X objective). Green dye shows Collagen type I in the extracellular matrix of the cells and blue dye shows nuclei of the cells.

A similar interpretation can be made for the expression levels of collagen type I on single layer PCL-PLLA scaffolds (Figure 5.11 a,b). The expression of collagen type I molecules at day 24 on single layer scaffolds was higher than that of at day 14. The amount of collagen type I expression on double layer PCL-PLLA scaffolds was slightly more than the ones on single layer PCL-PLLA scaffolds after 14 and 24 days of incubation.

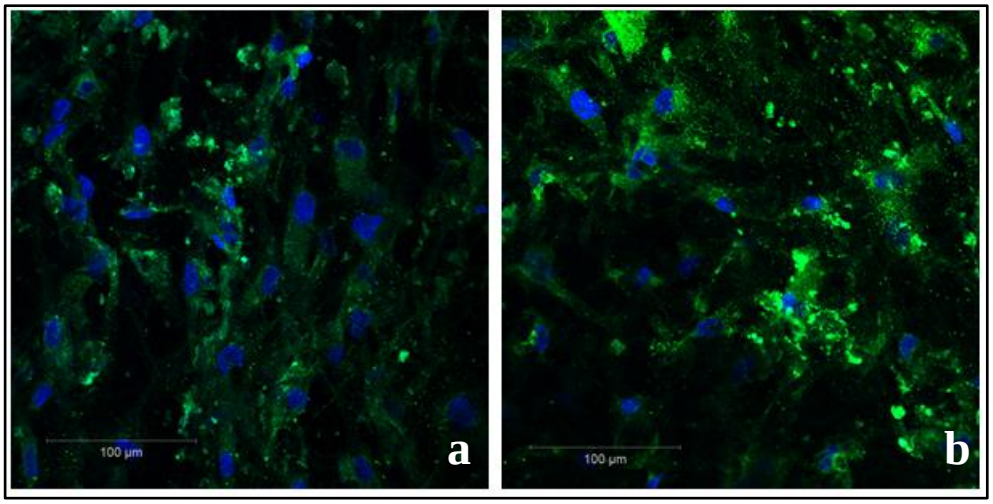

Figure 5.11. Confocal microscopy images of cells seeded on single layer PCL-PLLA scaffolds after (a) 14, (b) 24 days of incubation (with 20 X objective). Green dye shows Collagen type I in the extracellular matrix of the cells, blue dye shows nuclei of the cells.

At the second part of the experiment, the late marker osteocalcin was stained with Alexa Fluor® 488 dye for the determination of the late marker osteocalcin. It was observed that, the hADMSCs seeded on double layer scaffolds produced more osteocalcin protein at day 27 (Figure 5.12 b) when compared to the osteocalcin production at day 20 (Figure 5.12 a). A similar interpretation can be made for the expression levels of osteocalcin on single layer PCL-PLLA scaffolds (Figure 5.13 a, b).

The confocal microscopy analysis revealed that all of the scaffolds were supported the osteogenic differentiation of hADMSCs because they have expressed osteogenic markers on each of the scaffold.

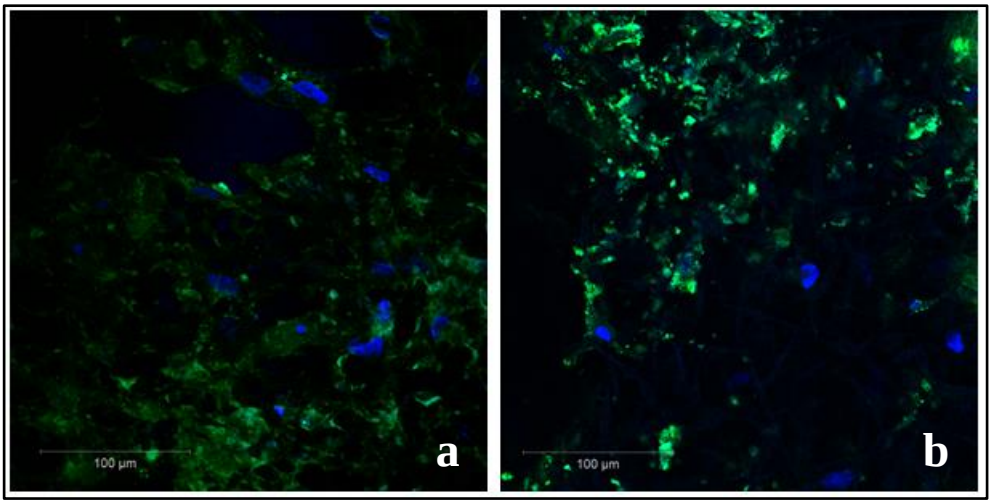

Figure 5.12. Confocal microscopy images of cells seeded on double layer PCL-PLLA scaffolds after (a) 20, (b) 27 days of incubation (with 20 X objective). Green dye shows osteocalcin in the extracellular matrix of the cells, blue dye shows nuclei of the cells.

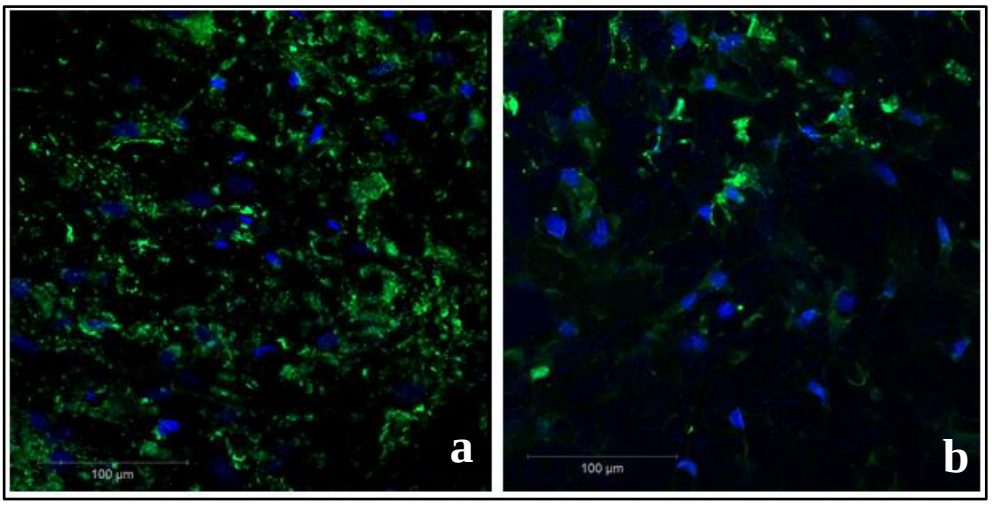

Figure 5.13. Confocal microscopy images of cells seeded on single layer PCL-PLLA scaffolds after (a) 20, (b) 27 days of incubation (with 20 X objective). Green dye shows osteocalcin in the extracellular matrix of the cells, blue dye shows nuclei of the cells.

6. DISCUSSION

Fabrication of scaffolds for tissue engineering is a demanding process requiring favorable and supportive properties for cell attachment, proliferation and differentiation. In order to examine these properties of the PCL-PLLA electrospun scaffolds MTS cell proliferation assay, ALP assay, Von Kossa Staining and immunocytochemical staining were performed.

Human adipose tissue was used as the source of mesenchymal stem cells. The characterization of stem cells was analyzed via flow cytometer at passage 3. The flow cytometer results were revealed that cells isolated from human adipose tissue were CD $^{90+}$, CD 44 $^{+}$ (mesenchymal stem cell markers) and CD 24^{-}, CD117^{-} (hematopoietic stem cell markers). Several studies examined the osteogenic differentiation potentials of hADMSCs and mesenchymality of the stem cells isolated from different sources. Wagner *et al.* compared the mesenchymal characteristics of the stem cells isolated from human bone marrow, adipose tissue, and umbilical cord blood. Although they showed some differences in the gene expression profiles of mesenchymal stem cell specific genes, the cell surface marker expression was similar in all sources and all of the MSC populations differentiate into osteogenic cells under the same conditions [110]. On the other hand, Im *et al.* showed that hADMSCs may have less potential for osteogenesis than human bone marrow derived mesenchymal stem cells (hBMMSC) when they were treated with the same amount of biological stimulators [111]. Conversely, De Ugarte *et al.* have not observed any significant differences between hADMSCs and hBMMSCs in terms of cell yield, cell senescence, growth kinetics and multi-lineage differentiation capacity [112]. Moreover, osteogenic differentiation capacity, ease of isolation with minimally invasive procedure, rapid proliferation and the abundance of the cell quantity make hADMSCs suitable and preferable source of MSC for bone tissue engineering.

The structure of the scaffold system was examined via SEM analysis. Li *et al.* showed that the best cell proliferation occurred on random fibers with the average diameter of 1,150 nm [113]. Kwon *et al.* proved that the endothelial cells were attached and proliferated well on PCL-PLLA copolymer fibers having 1,200 nm diameters [114]. These studies supported

our results since the fiber diameters of our double layer scaffold system (~1,000 nm) were close to the diameters of the fibers that were used in these studies.

MTS cell proliferation assay approved that both PCL-PLLA double layer and PCL-PLLA single layer scaffolds supported hADMSCs attachment onto scaffolds. Besides they have no cytotoxic effects on cells meaning that scaffolds provided a viable environment for cells. Cells seeded on double layer PCL-PLLA scaffolds had an increasing cell number profile when compared to the number of cells on single layer scaffolds. This situation indicates that cells proliferated on double layer PCL-PLLA scaffolds better than that on single layer PCL-PLLA scaffolds. In this study PCL and PLLA polymers were blended with weight ratios 1:1, similarly Chen *et al.* were fabricated a nanofibrous PCL-PLLA blend scaffold and investigated its biocompatibility with hADMSCs. They found that the blend ratio of 1:1 (w/w) is convenient for the electrospinning process and the scaffold favored the hADMSCs` attachment, viability, proliferation and osteogenic differentiation [115]**.**

ALP assay was performed to understand whether the cells were differentiated into osteogenic cells on PCL-PLLA electrospun scaffolds or not. At first, relative ALP activity levels of the cells on double layer and single layer PCL-PLLA scaffolds were investigated. Relative ALP activity levels were normalized to protein content of the cells in order to detect actual ALP levels. The ALP activity of the cells seeded on single layer PCL-PLLA scaffolds were rapidly increased (~20 fold) from day 1 to day 7 then decreased 1.5 fold at day 14. This decrease might be expected since ALP is an early marker for bone differentiation. For double layer PCL-PLLA scaffolds ALP activity levels were increased gradually from day 1 to day 14 (~5) as it was expected since the scaffolds contain BMP-2 loaded gelatin microspheres and the differentiation factors were released slowly in time. Park *et al.* examined the effect of BMP-2 loaded nanoparticles on osteogenic differentiation of human mesenchymal stem cells seeded on fibrin hydrogels. They observed higher ALP activity in the BMP-2 loaded nanoparticle containing hydrogels than the ones loaded either with BMP alone without nanoparticles or without BMP-2 and nanoparticles [116]. Shen *et al.* showed thatBMP-2-loaded poly(lactide-co-glycolide)/hydroxyapatite composite microspheres increased the ALP activity of mouse osteoblast like cells from day 1 through day 14 [117]. Differently, Kim *et al.* tested the

enhancement of early osteoblastic differentiation via sequential delivery of BMP-2 and insulin-like growth factor 1 (IGF-1). The delivery of BMP-2/IGF-1 subsequent to BMP-2 provided the highest ALP activity of osteoblast precursor cells. On the other hand, IGF-1 did not have any effect on osteogenic differentiation or osteoblast precursor cell proliferation [118]. Moshaverinia *et al.* encapsulated anti-BMP-2 monoclonal antibodies with mesenchymal stem cells in alginate microspheres to promote osteogenic differentiation of human mesenchymal stem cells [119]. When they introduced BMP-2 onto cells ALP was highly produced at 14^{th} day of incubation. In another study, a similar multilayer scaffold system was constructed. The system was prepared with poly(allylamine hydrochloride) (PAH) and poly(sodium 4-styrenesulfonate) (PSS) using layer-by-layer deposition technique. BMP-2 was adsorbed and diffused into the films between layers. The ALP activity of the mesenchymal stem cells on BMP-2 adsorbed multilayer films was greater than cell seeded films in BMP-2 containing medium [120]. All of these studies indicated that BMP-2 has a really significant effect on ALP expression and osteogenic differentiation of mesenchymal stem cells. Also the administration of BMP-2 with gelatin microspheres seems more effective than BMP-2 alone within the media. Therefore, the results of these studies in the literature are consistent with the presented study since the hADMSCs seeded on BMP-2 loaded microspheres consisting double layer scaffolds were expressed higher amount of ALP than the cells seeded on single layer PCL-PLLA scaffolds at day 14.

In Von Kossa staining experiment, mineralization was observed on both types of the scaffolds. The method was used to stain calcium deposits brown. Nuclear Fast Red dye stains nucleic acids in cells. At day 7 hADMSCs on double layer PCL-PLLA scaffolds were stained red and brown staining could not be observed since the mineralization had not been initiated. The calcium deposits started to accumulate at day 14 but the highest accumulation was observed on 24^{th} day of incubation. Throughout day 24, red regions on the scaffolds were diminished and brown regions were expanded. Increase in mineralization and decrease in cell proliferation can be inferred from this observation. The ColorPic. software was used to substantiate these findings. It determines the color dimensions: value, hue and saturation. The value dimension indicates lightness of a color. In our staining method, the value of the brown color was detected in order to quantify darkness of the calcium deposits. The numerical representation of the value is inversely

proportional with the darkness of a color, meaning the higher the value the lower the darkness. According to this knowledge, cells seeded on PCL-PLLA double layer scaffolds produced higher calcium deposits than cells on single layer scaffolds.

Expression of collagen type I and osteocalcin ECM proteins by hADMSCs was observed on both types of the scaffold via immunfluorescence analysis. PCL-PLLA blend nanofibrous scaffolds provided a suitable 3-D matrix for the attachment and proliferation of hADMSCs. They are widely studied FDA approved biopolymers and have no cytotoxic effects on cells as it was proved by MTS analysis. Collagen type I expression was increased from day 14 to day 24 on PCL-PLLA blend scaffolds. While the cells were adjacent to each other at day 14, they got further away from each other after 24 days of incubation. This situation may be explained by the increasing collagen type 1 production by cells and expansion of the ECM after 24 days of incubation. Apart from these, Kihara *et al.* showed the effect of collagen type I on osteogenic differentiation of rat MSCs by adding collagen type I exogenously. They discovered that collagen type 1 stimulates the osteogenic differentiation and matrix mineralization [121].

Osteocalcin is a bone matrix protein secreted by osteoblasts at later stages of bone development. hADMSCs expressed osteocalcin on both single and double layer PCL-PLLA scaffolds. The amount of the osteocalcin produced on the double layer scaffolds increased from day 20 to day 27. Nguyen *et* al. investigated the osteogenic differentiation of human MSCs on electrospun PLLA scaffolds that were loaded with dexamethasone. They found that osteoblastic gene expression (ALP and osteocalcin) increased towards day 21 and the highest gene expression detected on PLLA scaffolds without dexamethasone [122]. Tian *et al.* examined the biocompatibility of different types of nanofibrous scaffolds (poly(L-lactic acid-co-ϵ-caprolactone) (PLCL)/hydroxyapaptite (PLCL/HA), PLCL/laminin (PLCL/Lam), and PLCL/hydroxyapatite/laminin (PLCL/HA/Lam)). The results of the study indicated that the osteoblasts were able to adhere, proliferate and showed augmented ALP activity, bone protein expression and mineralization [123].

In accordance with these studies and results of this research we can conclude that our scaffold system was perfectly suitable for the attachment, proliferation, differentiation and

maturation of the hADMSCs. Intercalarily, the double layer scaffold system might be improved by blending PCL-PLLA with some other polymers or some surface treatments.

7. CONCLUSION

The main purpose of this research was to investigate biocompatibility and osteoinductive capacity of growth factor loaded microsphere containing double layer PCL-PLLA nanofibrous scaffold system in order to reveal whether it can be a good candidate for bone tissue engineering or not.

PCL is a hydrophobic polymer, characterized with good permeability and rubbery structure; in contrast PLLA is brittle and tough. When these two polymers are combined, they create a new polymer composite that is mechanically strengthened. Furthermore, the electrospinning technique was used for the fabrication of the scaffold system to mimic the natural fibrous mesh structure of bone.

hADMSCs were used to examine the scaffold system. They were seeded on nanofibrous single/double layer PCL-PLLA scaffolds. MTS assay showed that cells were able to attach and proliferate on both of the scaffolds. This result indicated that the scaffolds do not have any cytotoxic effect on hADMSCs, therefore they are biocompatible.

ALP assay and Von Kossa staining demonstrated that double layer PCL-PLLA scaffolds have higher osteoinductive capacity than that of single layer scaffolds. At day 14, cells seeded on double layer scaffolds synthesized more ALP than cells on single layer scaffolds. Likewise, higher mineralization was observed on double layer PCL-PLLA scaffolds at day 24. For this reason, this scaffold system may have significant potential for bone regeneration applications. Moreover, the observations made through confocal microscopy studies supported the results obtained from ALP assay and Von Kossa staining.

In conclusion, double layer PCL-PLLA scaffolds promoted hADMSC attachment, growth, proliferation and differentiation. Biocompatibility and the osteoinductive capacity of the double layer PCL-PLLA scaffolds have been proven via this study. This scaffold system is a promising candidate for tissue engineering applications.

8. FUTURE PROSPECTS

In order to improve our scaffold system, different polymers can be used to create the 3-D scaffolds. For example, since PCL and PLLA are hydrophobic materials, top layer of the scaffold can be mixed with hydrophilic polymers such as cellulose, polyethylene glycol ethers, polyamides or polyacrylic amides. Surface modifications might be done to increase biocompatibility of the materials. Co-administration of BMP-2 with its heterodimers BMP-4, BMP 7 or BMP-9, or sequential delivery of BMP-2 and IGF-1 can be tried to induce osteogenic differentiation. Additionally, growth factor release kinetics can be tailored with altering the microsphere material (for example using alginate instead of gelatin) and changing the dosage or concentration of the growth factors.

Further *in vivo* experiments should be done to designate systemic effect and inflammatory response of the scaffold system on the body. For this purpose, double layer PCL-PLLA scaffolds should be tested primarily in animal models.

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APPENDIX A

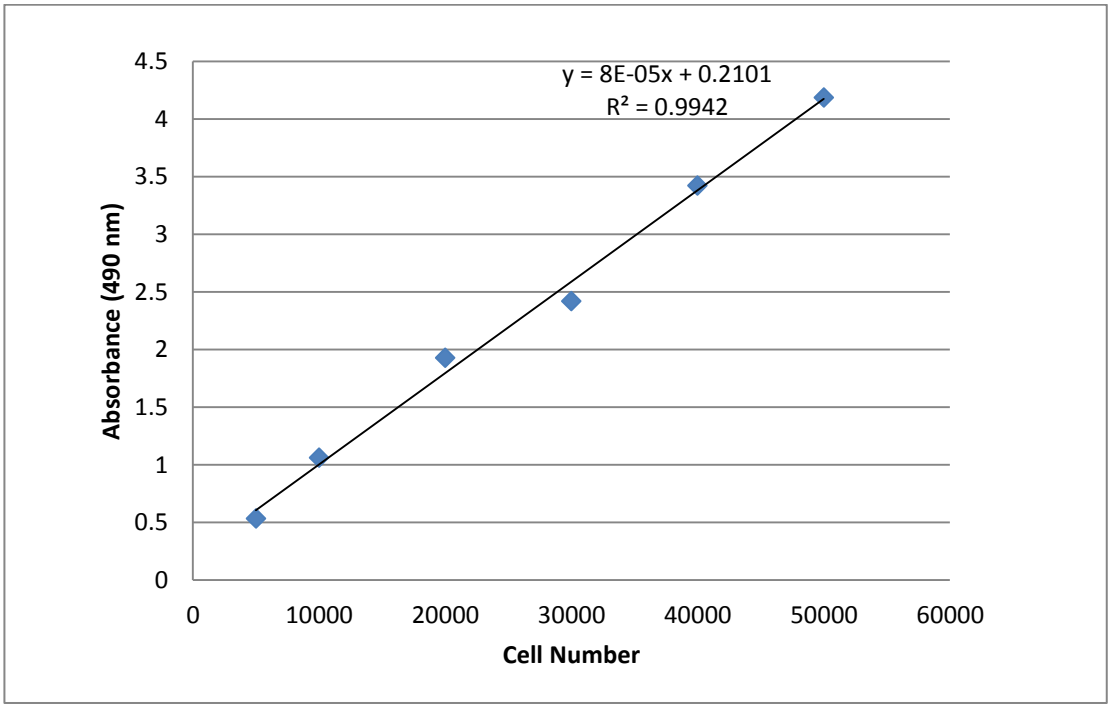

Figure A.1. Calibration curve for hADMSCs (blue quadrangles) and its trendline (black). (Equation of the slope is shown on the graph)