**ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY**

# USE OF pH AND TEMPERATURE SENSITIVE NANOSPHERES IN CARTILAGE TISSUE ENGINEERING

**M.Sc. Thesis by**
**Ayşe Burcu Ertan, B.Sc.**
**(521051222)**

**Department: Advanced Technologies**
**Programme: Molecular Biology, Genetics and Biotechnology**

**Supervisors : Assist. Prof. Fatma Neşe Kök (ITU)**
**Assoc. Prof. Gamze Torun Köse (YU)**

**JANUARY 2009**

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**Assoc. Prof. Dr. Gamze Torun Köse (YU)**

**Members of the Examining Committee:** **Prof.Dr. Vasıf Hasırcı (METU)**

**Assoc. Prof. Dr. Ayten Yazgan Karataş (ITU)**

**Assoc. Prof. Dr. Zeynep Petek Çakar (ITU)**

**JANUARY 2009**

**İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ**

# pH VE SICAKLIĞA DUYARLI NANOKÜRELERİN KIKIRDAK DOKU MÜHENDİSLİĞİNDE KULLANIMI

**Yüksek Lisans Tezi**
**Ayşe Burcu Ertan**
**(521051222)**

**Tezin Enstitüye Verildiği Tarih : 26 Aralık 2008**
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| | |
|---|---|
| **Tez Danışmanı :** | **Yrd. Doç. Dr. Fatma Neşe Kök (ITU)** |
| | **Doç. Dr. Gamze Torun Köse (YU)** |
| **Diğer Jüri Üyeleri** | **Prof.Dr. Vasıf Hasırcı (ODTU)** |
| | **Doç. Dr. Ayten Yazgan Karataş (ITU)** |
| | **Doç. Dr. Zeynep Petek Çakar (ITU)** |

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BSc Ayşe Burcu Ertan
Molecular Biologist

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**ABBREVIATIONS**

| | |
|---|---|
| **Ab** | : antibody |
| **CHA** | : Chondroitin sulphate and alginate |
| **ColII** | : Collagen type II |
| **CS** | : Chondroitin sulfate |
| **DCM** | : Dichloromethane |
| **DMEM** | : Dulbecco's Modified Eagle's Medium |
| **DMMB** | : (1,9-dimethylmethylene blue) |
| **ECM** | : Extracellular matrix |
| **EP** | : differentiation medium with empty PLGA nanospheres |
| **EN** | : differentiation medium with empty NIPAM nanospheres |
| **FBS** | : Fetal bovine serum |
| **sGAG** | : sulphated glycosaminoglycan |
| **IGF-I** | : Insulin-like growth factor- I |
| **IP** | : differentiation medium with IGF-I loaded PLGA nanospheres |
| **IPTN** | : differentiation medium with IGF-I loaded PLGA nanospheres + TGF-β1 loaded NIPAM nanospheres |
| **MTS** | : 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium |
| **NIPAM** | : N-isopropylacrylamide |
| **OC** | : only differentiation medium |
| **PBS** | : Phosphate-buffered saline |
| **PLGA** | : Poly(lactic acid-co-glycolic acid) |
| **SEM** | : Scanning Electron Microscope |
| **TCPS** | : Tissue Culture Polystyrene |
| **TGF-β1** | : Transforming growth factor-beta |
| **TN** | : differentiation medium with TGF-β1 loaded NIPAM nanospheres |
| **Trypsin-EDTA** | : Trypsin - Ethylenediaminetetraacetic acid |

## USE OF pH AND TEMPERATURE SENSITIVE NANOSPHERES IN CARTILAGE TISSUE ENGINEERING


### SUMMARY

The developments in science and technology prolong the life span of the human beings. Long life brings with it problems associated with degenerative diseases and worn out organs. Tissue engineering aims at repairing diseased or nonfunctional organs through the design and use of cell carrying biodegradable biomaterials. The cell sources available for cartilage tissue engineering are mainly chondrocytes and mesenchymal stem cells. Upon use of appropriate growth factors mesenchymal stem cells are known to differentiate into a variety of cells such as bone, cartilage, fat and muscle cells.

In this study, the aim is to obtain cartilage regeneration through differentiation of mesenchymal stem cells. The growth factor choice is crucial for a successful result. During the damage and repair of joint cartilage a variety of growth factors such as insulin-like growth factor I (IGF-I), and transforming growth factor beta1 (TGF-β1) are released. Knowing that introduction of TGF-β1 on chondrocytes increases cell proliferation and IGF-I induces chondrocyte phenotype in 3D *in vitro* cultures, both growth factors are studied for their effect as controlled release formulation

Controlled release of IGF-I and TGF-β1 were realized from two different polymeric nanospheres, namely PLGA and NIPAM. The nanospheres were introduced to PLGA based cell carriers and the effect of their release profile on cell proliferation and differentiation was studied. At the end of the experiments, it can be concluded that cells treated with IGF-I and IGF-I / TGF-β1 containing nanospheres give the best results with respect to cartilage differentiation onto scaffolds. It thus appears that, usage of growth factor loaded nanospheres could have a serious contribution to cartilage tissue engineering.

## pH VE SICAKLIĞA DUYARLI NANOKÜRELERİN KIKIRDAK DOKU MÜHENDİSLİĞİNDE KULLANIMI


## ÖZET

Bilim ve teknolojideki gelişmeler insanların yaşam süresini uzatmaktadır. Uzun yaşam beraberinde dejeneratif hastalıklar ve yıpranan organlarla ilgili sorunları getirmektedir. Doku mühendisliği hasar görmüş ya da fonksiyonlarını yitirmiş organların tedavisi amacıyla hücre taşıyan biyobozunur malzemelerin tasarımlarını ve uygulanmalarını hedeflemektedir.

Kıkırdak doku mühendisliğinde kullanılabilecek hücre kaynakları temelde kondrositler ve mezenşimal kök hücreleridir. Uygun büyüme faktörleri kullanıldığında, mezenşimal kök hücreleri kemik, kıkırdak, yağ ve kas gibi değişik dokulara dönüşebilmektedir. Bu çalışmada mezenşimal kök hücrelerin kıkırdak hücrelerinin kondrositlere dönüşmesi ve kıkırdak rejenerasyonunu başlatması sağlanmaya çalışılmıştır. Bu aşamada büyüme faktörlerinin tipi büyük önem taşımaktadır. Eklem kıkırdağının hasarlanması ve onarılması sırasında Insülin Benzeri Büyüme Faktörü - I (IGF-I), ve Transforming Büyüme Faktörü Beta1 (TGF-β1) gibi bir çok büyüme faktörü salınmaktadır. Kıkırdak hücreleri üzerine TGF-β1 eklenmesi kondrosit çoğalmasını teşvik etmekte, IGF-I ise 3 boyutlu in vitro kültürlerde kondrosit fenotipini artırmaktadır.

Bu çalışmada bu iki büyüme faktörü ve hücreler ayrı ayrı PLGA temelli akıllı (responsif) hücre taşıyıcılar içine yüklenmiş ve pH ile sıcaklığa bağlı olarak birbirini izleyen zamanlarda salımları sağlanarak hücrelerin çoğalması ve farklılaşması sağlanmıştır. Yapılan deneyler sonunda IP ve IPTN ile birlikte bulunan hücrelerin, iskelet yapılar üzerinde kıkırdak farklılaşması açısından en iyi sonuçları verdiği gözlemlenmiştir. Böylece, büyüme faktörü yüklü nanokürelerin kıkırdak doku mühendisliğinde ciddi katkıları olabileceği görülebilmektedir.

# 1. INTRODUCTION

## 1.1. Articular Cartilage

Articular cartilage essentially provides mechanical support for the functioning of diarthrodial joints that are found between bones. In a living joint, cartilage can hold up against a great pressure which can be up to 20- fold body weight, therefore, it specifically functions in avoiding the possible subchondral bone damages (Plumb and Aspden et al., 2005; Li et al., 2008). In comparison with bone and muscle, cartilage is known to show lower metabolic activity and limited damage healing. Its macroscopic surface properties include smoothness, glistening and tough material appearance. Furthermore, cartilage is composed of mostly extracellular matrix (ECM) and does not include any blood and lymph vessels or nerve ends. Moreover, endoplasmic reticulum and golgi apparatus are the most distinguishable organelles of the cartilage cell with the light microscope and many of them contain lipid and glycogen stocks, and also vesicles functioning in secretion (Dedivitis et al., 2004; Temenoff et al., 2000).

The non-homogenous tissue characteristic of articular cartilage comes from the fact that in humans, cartilage cells, known as chondrocytes, represent only about 1% of the volume of hyaline cartilage. In spite of their small volume, chondrocytes are essential in replacing degraded matrix molecules so that tissue can maintain its usual size and mechanical abilities (Darling et al., 2005; Temenoff et al., 2000). Additionally, secretion and maintaining of ECM that have a part in growth, mechanical support and the function of diarthrodial joints, are among the specific functions of chondrocytes (Yang et al., 2005).

Cartilage, located between joint cavities to subchondral bone, can be divided in 4 zones: superficial, middle, deep and calcified (Figure 1.1). The superficial zone consists of two distinct layers. First layer is described as the cover of the joint and it consists of a sheet composed of predominantly collagen fibers with a little polysaccharide content without any cells. Under this sheet, chondrocytes can be

found layered in a flattened and parallel manner to the surface that constitutes the second layer. The collagen fibrils are also arranged parallel to the articular surface in this zone.

In the middle zone (or transitional), round chondrocytes are surrounded by a narrow pericellular region of low collagen fibril content (Temenoff et al., 2000; Poole et al., 2001). This zone consists of the highest level of proteoglycan and it has a random arrangement of collagen.

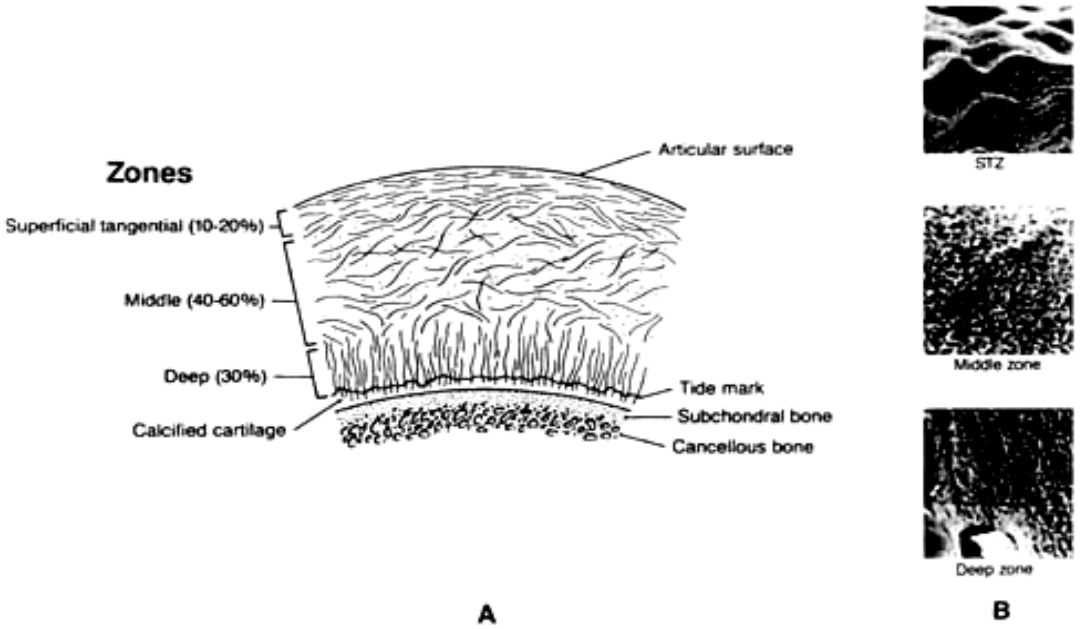


**Figure 1.1.** Schematic representation of articular cartilage (Mow and Ratcliffe, 1997)

The deep zone has the lowest cell density, but it shows maximal aggrecan content, also consists of large and spherical cells clustered in column. Columns of chondrocytes can be observed as arrayed along the axis of fibril orientation, and additionally, collagen fibrils are oriented in a vertical pattern, perpendicular to the bone (Mow and Ratcliffe., 1997).

The calcified zone is partly mineralized and lies closest to the subchondral bone. It acts as a transition between cartilage and the underlying subchondral bone. As a consequence of being the interface between the soft cartilage and much harder bone, significant shear stress can be produced in this calcified zone. The local chondrocytes of this zone are smaller; they have almost no endoplasmic reticulum and structurally seem to be surrounded by calcified ECM, therefore having almost no space for metabolic activities (Cohen et al., 1998; Temenoff et al., 2000).

## 1.2. Molecular Components of Articular Cartilage

ECM consists of collagens, proteoglycans, and non-collagenous proteins (DiCesare et al., 1995). The primary structure of a collagen molecule is a long and tight triple-stranded helix, in which three collagen polypeptide chains, $[\alpha 1(II)]_3$ ($\alpha$ chains), are tied around one another (Alberts et al., 2002). In macromolecular framework, collagens (especially type II) are responsible in the physical entrapment of the other macromolecules and in creating tensile strength against stretching. While proteoglycans, composed of about 95% polysaccharide and 5% protein, provide some physical properties to the tissue. Finally, non-collagenous proteins affect the stabilization of the ECM matrix and help building interactions between chondrocytes and matrix.

In addition to abundantly found type II collagens (85-90% of the total collagen content), collagen types VI, IX, X and XI are found in articular cartilage. Differing from other type of collagens, type II collagen is highly responsible for interaction with water since it consists of a high amount of bound carbohydrate groups. Collagen type VI is a glycoprotein with a short collagenous central domain. Being 5 nm in diameter, its supramolecular structure has 100 nm periodicity (Meyer and Wiesmann, 2006). Types IX and XI together with type II create fibrils that twine together to form a meshed structure which provides the tensile strength, and also physical entrapment of the other macromolecules (Temenoff et al., 2000). Collagen type IX is found on the external part of the collagen fibrils, allowing the fibrils to interact with proteoglycan macromolecules, which stabilizes the meshed structure. It is a member of subfamily of collagens, FACIT (Fibril Associated Collagens with Interrupted Triplehelices), and has a molecular weight of 222 kDa. Collagen types IX and XI constitute 3-10% of the collagen content, depending on age and the source of cartilage. Collagen type X is located in the calcified area of articular cartilage (Ackermann and Steinmeyer, 2005). There are two types of collagen fibrils: thin (16-nm diameter) and thick (~40-nm diameter). The cores of thin fibrils are particularly composed of collagen type XI (Figure 1.2), a fibrillar collagen type with 545 kDa weight (Meyer and Wiesmann, 2006; Kadler et al., 2008).

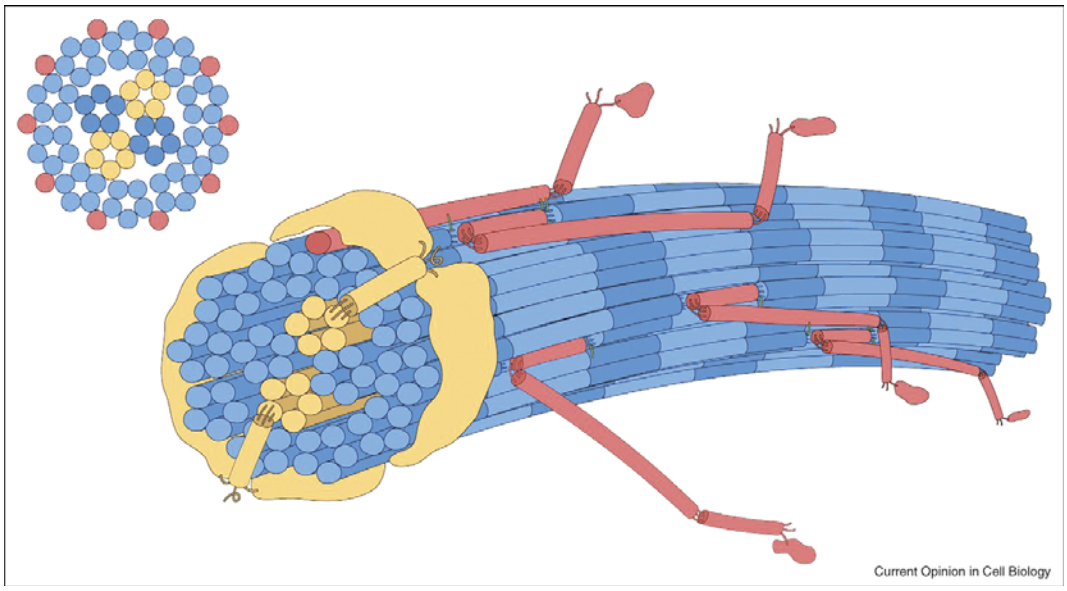


**Figure 1.2.** Microfibril structure of a thin collagen fibril (Blue: collagen II molecules; yellow: collagen XI molecules; red: collagen IX molecules). A pair of collagen XI microfibrils comprises half of a 4 microfibril core surrounded by 10 microfibrils at the surface. The collagen XI/IX/II assembly is a crosslinked heteropolymer (Kadler et al., 2008).

Predominant collagen of cartilage and intervertebral disc is collagen type II that provides tensile strength and enables the tissue to resist shearing forces (Meyer and Wiesmann, 2006). Being a triple helical glycoprotein with a molecular weight of 425 kDa, collagen type II possesses a fibrillar structure with 67 nm periodicy. Collagen type II is produced and then secreted by chondrocytes by exocytosis, supporting chondrocyte adhesion and phenotypic differentiation of cells. It contains specifically proline and lysine amino acids and many modifications that are almost unique to collagen. These modifications such as hydroxylation of residues are introduced during biosynthesis eventually creating hydroxyproline and hydroxylysine (Stryer, 1981). The high hydroxylysine content of collagen type II, facilitates glycosylation (a property of hydrophilic tissue), makes collagen type II the most suitable component of cartilage (Ackermann and Steinmeyer, 2005). The abundance and structures of collagen molecules vary according to the anatomy and age of the person (Meyer and Wiesmann, 2006).

Aggrecan, a non-collagenous protein with 87% chondroitin sulphate (CS) and 6% keratan sulfate (KS) side chains, is another essential component of cartilage tissue (Figure 1.3). It is a large proteoglycan having a molecular weight of 205 kDa, and consists of three globular domains (G1, G2, G3) with glycosaminoglycan (GAG)

attachment regions for KS an CS. Forming complexes with hyaluronan through G1 domain, it belongs to the family of aggregating proteoglycans, hence the name is "aggrecan" (Gomes et al., 2004). The large aggregates formed by aggrecan and hyaluronan (>200 MDa) provide the maintenance of aggrecan within the ECM (Figure 1.4). Due to the presence of large number of negatively charged side groups, it provides resistance to compression. The primary role of aggrecan is to swell and hydrate collagen framework, making high osmotic pressure in order to reinforce cartilage. Studies have shown that aggrecan coats collagen type II, preventing it from proteolytic degradation. Without aggrecan molecules, collagen fibers are not supported well, thus the tissue is open to damaging forces (Pratta et al., 2003; Meyer and Wiesmann, 2006; Kuno et al., 2000; Kamarainen et al., 2006).

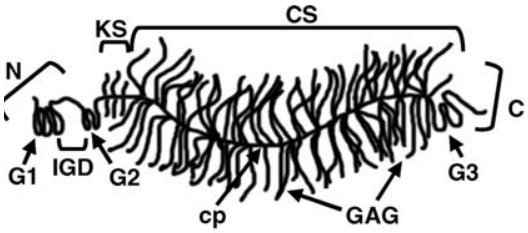


**Figure 1.3.** Structure of aggrecan: N: amine-terminal, G1, G2, G3: globular domains, IGD: interglobular domain between G1 and G2, cp: core protein, KS: keratan sulfate region, CS: chondroitin sulfate brush region, GAG: glycosaminoglycan chains, C: carboxyl-terminal (Ng et al., 2003).

### 1.3. Disorders

As mentioned before, articular cartilage can hold against a dramatic amount of mechanical stress, however, it has a very restricted capability of self-repair when it encounters a destructing trauma (Hunziker and Rosenberg, 1996; Hunziker, 1999, Buckwalter and Mankin, 1998).

In Europe and North America, the major cause of disabilities is the osteoarticular disorders. Moreover, rheumatoid arthritis is very common among the populations, effecting about 1% of individuals. It is very abundant, affecting more than two third of people over age 55 (Gouze et al., 2007). More dramatically, osteoarthritis can be found to strike even more than one third of the adult population in industrialized countries (Spagnoli et al., 2007).

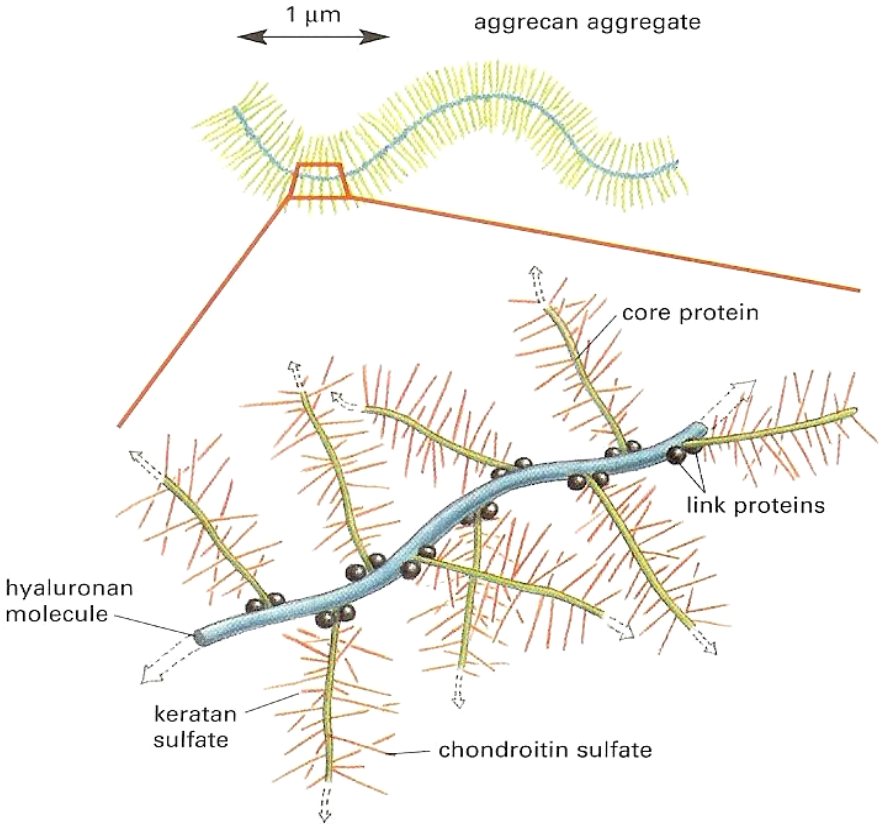


**Figure 1.4.** A drawing of aggrecan aggregate: About 100 aggrecan monomers are noncovalently bound to a single hyaluronan chain by two link proteins. The link proteins are members of a family of hyaluronan-binding proteins, some of which are cell-surface proteins. This type of complex can have a molecular weight of $10^8$ >, occupying a volume of 2 x $10^{-12}$ $cm^3$, which is equal to that of a bacterium (Alberts et al., 2002).

Osteoarthritis can be defined as disruption of collagen/proteoglycan network, leading to swelling and softening of the tissue. Developmental abnormalities, genetic disorders in EMC molecules, excessive usage or limb malposition, and metabolic problems, such as diabetes, alcoholism, and being overweighed are the causes of osteoarthritis. Furthermore, trauma, caused by direct injury to the joint or ligaments, commonly leads to osteoarthritis. Generally being an age-related degenerative joint disease, osteoarthritis was shown to be associated with low chondrocyte cell density, insufficient synthesis of ECM, and hypertrophy. Cartilage tissue engineering is mostly applied to trauma patients since the best data of human osteoarthritis were collected from those patients. Hopefully, the results

indicate considerably successful prevention or at least retardation of the disease (Mollenhauer, 2008; Wang et al., 2006).

Studies on cartilage degeneration have shown that aggrecan fragments generated by aggrecanase action were present in synovial fluids from normal individuals and patients with osteoarthritis, rheumatoid arthritis and acute knee injuries (Lohmander et al., 1993). As aggrecan molecules are degraded, they are no longer able to protect collagen fibers, which will cause proteolytic degradation of collagens. Therefore, the reason of articular cartilage degenerations is the proteolytic degradation of aggrecan and collagen (Sztrolovics et al., 1997). However, their rates of synthesis are increased as well, suggesting an activated repair mechanism that is not sufficient in repairing or maintaining the ECM homeostasis after all (Koevoet et al., 2008).

### 1.4. Tissue Engineering of Articular Cartilage

Tissue engineering is a promising new therapeutic approach that can overcome the problems of autographs allografts and xenografts. In case of cartilage tissue engineering, the critical part is the selection of appropriate cell type such that cells can be differentiated or progenitor. It essentially depends on fabrication and usage of biocompatible and mechanically stable scaffold for cell delivery, furthermore on stimulation by applying chondrogenically bioactive molecules that can be introduced either in the form of recombinant proteins or through transferring of genes. Moreover, application of dynamic, mechanical loading regimen under the ultimate aim of conditioning of the engineered tissue constructs that include specialized biomechanically active bioreactor design is among the critical dependences of cartilage tissue engineering. Therefore, selection of the cell type and scaffold, together with the construction of design and stimulation are the challenges in cartilage tissue engineering that should be overcome (Kuo et al., 2006).

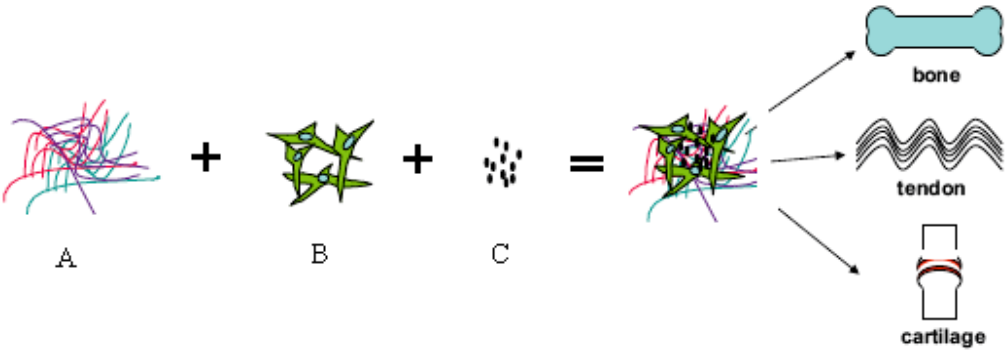


**Figure 1.5.** Basic overview of tissue engineering (Csaki et al., 2008). Three main factors are essential for tissue engineering: Initially, (A) a compatible biomaterial, (B) suitable cells, for instance stem cells, (C) specific bioactive substances, when found all together, they enhance adequate cell differentiation and specific tissue formation such as bone, tendon and cartilages.

Among the treatments of traumatic articular cartilage injuries, cartilage engineering has an applicable part. The most encouraging goal would be to regenerate destroyed cartilage due to osteoarthritis that is the most common type of musculoskeletal system diseases among individuals.

Tissue engineering requires a successful and efficient interaction between bioactive factors, cells, their extracellular environment, and a functional matrix or scaffold to provide a physical form to the structure (Figure 1.5).

In some tissue engineering application, the growth and differentiation factors are specifically offered together with a matrix material in order to create a promising environment for the host cells to populate. For instance, in other applications, *in vitro* treatment of exogenous cells with the growth factor before implantation or supplementation of the cells can be observed.

### 1.5. Cell Sources

In cartilage tissue engineering, xenogenic, autologous and allogenic cells are considered as the main cell sources. Those cell categories can be divided in subcategories according to their characteristics of being a stem cell or a more differentiated cell such as primary cells or cell lines. In bone and cartilage tissue engineering studies, many types of mature cell lines, such as multipotent mesenchymal progenitors are widely used. In other targets than humans, basic aspects of *in vitro* cell behaviors were evaluated using recently developed other

types of bone and cartilage cell lines like genetically altered cell lines (sarcoma cells, immortalized cells, non-transformed clonal cell lines) (Yamaguchi et al., 2002; Meyer and Wiesmann, 2006).

Mesenchymal stem cells (MSCs) that can be found in the bone marrow in mature individuals are responsible for generating chondrocytes. During the embryogenesis, when MSC differentiation into chondrocytes takes place, MSCs start to secrete a cartilaginous matrix while the cells keep up dividing (Figure 1.6).

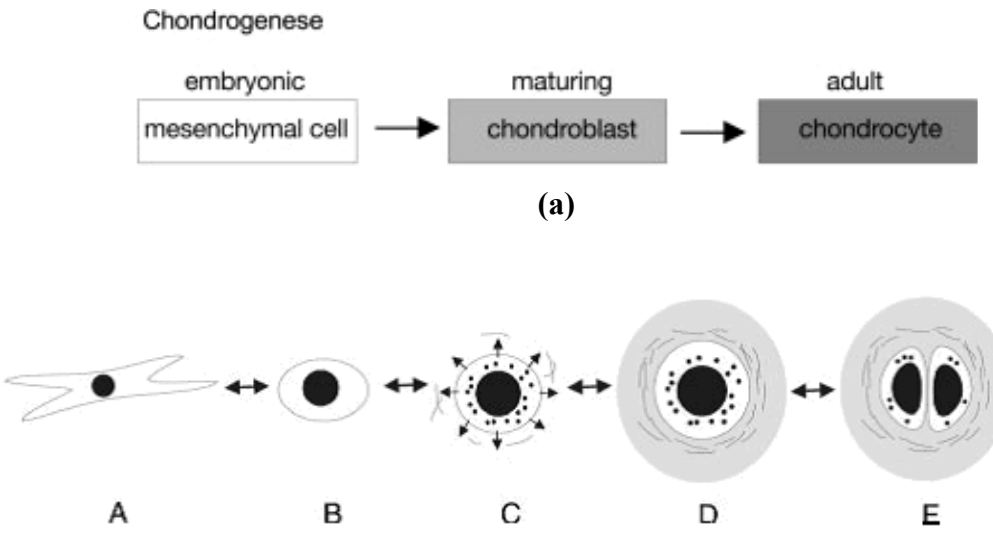


**Figure 1.6.** (a) Terminal differentiation of a chondrocyte: During natural development, a mesenchymal cell turns into a chondroblast and then into a chondrocyte, which is an irreversible process (b) Schematic illustration of the development of a mesenchymal stem cell into a chondrocyte: The development includes the transformation of a polymorphic mesenchymal cell (A) to a rounded chodroblast (B), which characterizes its own cavity, in interaction with the ECM (C). The number of chondrocytes that will share this lacuna and how the chondral capsule should be developed must be determined. According to these conditions, the mechanically loadable intercellular substance is then developed (D– E) (Minuth et al., 2005).

Primary chondrocytes, another cell source for cartilage regeneration, can be isolated from articular cartilage explants. When isolated, these chondrocytes dedifferentiate; in other words, stop expressing collagen type II and aggrecan, lose their characteristic phenotypes, which are round-shaped, adopt a fibroblastic shape, and start to proliferate. These chondrocytes can be induced to redifferentiate by

culturing them in a suitable environment that includes differentiation factors like TGF-β1. Cultured chondrocytes are less multipotent than MSCs; hence, it is less expected to generate unrelated cell types when these chondrocytes are used for the repair of cartilage tissue (Kaplan et al., 2006, Pittenger and Marshak, 2001).

Fibroblasts can easily be obtained in high numbers and be directed toward chondrogenic differentiation. French et al. (2004) have demonstrated that human dermal fibroblasts induced with IGF-1 and cultured on aggrecan formed dense aggregates that stain positive for GAGs and collagen type II. In addition, induced pluripotent stem (IPS) cells developed from adult human fibroblasts, and adipose-derived stem cells can be counted as cell sources for cartilage tissue engineering in the case of chondrocyte shortage (Wang et al., 2008).

### 1.6. Biomaterials

A biomaterial should be biodegradable or bioresorbable to permit the reconstruction of a normal tissue, and its degradation products should not provoke inflammation or toxicity if it is going to be used in tissue engineering. (Kim et al., 2000). It should be able to regulate cell behavior, which includes cell adhesion, growth, and differentiation, in order to promote development of a functional new tissue. In addition, the final configuration of the biomaterial should have a large surface area-to-volume ratio for better cell-polymer interactions, ECM regeneration, and for minimal nutrient and waste diffusion constraints. Finally, it should be reproducibly processable with appropriate mechanical properties, and its degradation rate should be adjustable to match the rate of tissue regeneration by the cells.

#### 1.6.1. Natural Biomaterials

Naturally derived macromolecules have potential advantages of biocompatibility, cell controlled degradability, and intrinsic cellular interaction (biological recognition). However, they may exhibit batch-to-batch variations and generally exhibit a narrow and limited range of mechanical properties (Lee et al., 2001). They can be obtained from various sources: ECM (collagen, hyaluronic acid) or plasma (fibrin) of mammalian tissues, crustaceans and seaweeds (chitin and alginate, respectively), microorganisms (polyhydroxyalkanoates).

### 1.6.2. Synthetic Biomaterials

In contrast to natural macromolecules, synthetic polymers can be prepared to have more precisely controlled structures and properties (Lee et al., 2001). They can be produced reproducibly on a large scale with relatively controlled strength, degradation rate, and microstructure, like porosity, by either altering the ingredients employed or polymer-processing methods (Marler et al., 1998). Properties of synthetic polymers can be altered by varying functional groups (backbone or side chain), polymer architecture (linear, branched, comb or star) and polymer combinations (polymer blends, interpenetrating networks or chemically bonded copolymers) (Wong et al., 1997). Poly (α-hydroxy acids) such as poly lactic acid (PLA) and its copolymers with glycolic acid (PLGA) are the most commonly used polymers in medicine besides other synthetic polymers like poly(anhydrides) and poly(ortho-esters).

PLA, PGA, and PLGA have gained FDA approval for human use for a variety of applications, including sutures. Since these polymers are thermoplastics, they can be easily formed into desired shapes by various techniques including molding, extrusion or by solvent casting. PGA, which has the simplest structure among linear aliphatic polyesters, is highly crystalline and thus has a high melting point and low solubility in organic solvents. PLA is more hydrophobic than PGA due to the presence of an extra methyl group in the structure of lactic acid and dissolves more readily in organic solvents (Pachence and Kohn, 1997).

The degradation rate of the copolymers can be tailored from several weeks to several years by alteration of their crystallinity, molecular weight, and the ratio of lactic to glycolic acid. However, it should be noted that there is no linear relationship between the ratio of glycolic acid to lactic acid and the physicochemical properties of their copolymers. Although PGA is crystalline, this feature is lost in PGA-PLA copolymers and degradation takes place more rapidly than either PGA or PLA. The ester bonds in these polymers are hydrolytically labile and these polymers degrade by nonenzymatic hydrolysis. PGA hydrolyses in water to glycolic acid, which can be converted enzymatically to glycine to be used in protein synthesis or pyruvate that will enter the tricarboxylic acid cycle and eventully eliminated from the body in the form of carbon dioxide and water. PLA

also undergoes hydrolytic deesterification into lactic acid, which becomes incorporated into the tricarboxylic acid cycle to be converted to $CO_2$ and $H_2O$.

Linear aliphatic polyesters such as PLA, PGA, and copolymers (PLGA) are synthetic biocompatible polymers that are approved by Food and Drug Administration (FDA) and therefore they are widely used (Figure 1.7). Their rate of biodegradation and mechanical characteristics may be altered simply by changing their copolymer ratio. Moreover, they are proved to be applicable in many tissue engineering applications, and also are used in drug delivery studies based on the fact that their degradation products, formed as a result of their hydrolysis, can be naturally found as parts of metabolic pathways in human body (Lo et al., 1996; Richardson et al. ,2001; Jang and Shea, 2003; Nof and Shea., 2002 Sengupta et al., 2005; Freed et al., 1993; Sarazin et al., 2004).

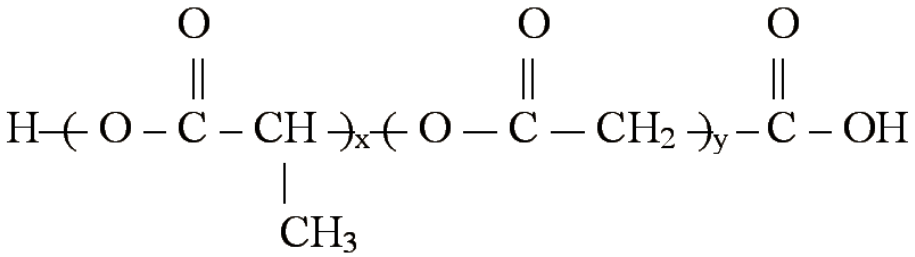

**Figure 1.7.** The structure of poly (lactic-*co*-glycolic acid) (PLGA).

N-isopropylacrylamide (NIPAM) has been one of the most commonly studied thermoreversible synthetic biomaterial. The polymers exhibit a solubility change as a function of temperature, which could be utilized for convenient delivery of proteins and cells (Jiang et al., 2006).

It forms a three-dimensional hydrogel when crosslinked with N,N'-methylene-bis-acrylamide (MBAm) or N,N'-cystamine-bis-acrylamide (CBAm). When heated in above 33°C, it undergoes a reversible phase transition from a swollen hydrated state to a shrunken dehydrated state, losing about 90% of its mass. Since PNIPAm expels its liquid contents at a temperature near that of the human body, PNIPAm has been investigated by many researchers for possible applications in controlled drug delivery (Chung et al., 1999).

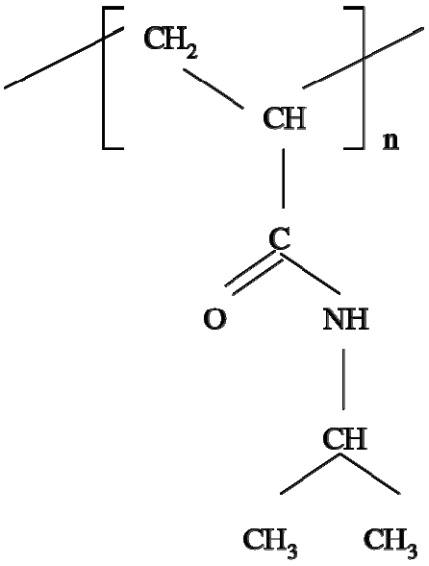


**Figure 1.8** Formula of a single NIPAM molecule (Chung et al., 1999).

### 1.7. Biological stimulation

Growth factors, found in polypeptidic structures, have roles in the cellular communication system (Nimni, 1997). Their action is based on signal transmission under the ultimate aim of modulating cellular activity by either inhibiting or enhancing proliferation, differentiation, migration, or gene expression (McKay and Leigh, 1993). In general, growth factors exhibit pleiotropic behaviors, such that same growth factor may have different cells as targets in order to create similar or distinct effects. Furthermore, another effect called redundancy is a part of growth hormone properties, meaning that they can exert the same effect for a specific cell type. Their exhibition of stimulation or inhibition can be either through an endocrine (released directly into the blood stream), paracrine (diffusion to neighbor target cell) or autocrine (source and target cell are the same) fashion. Their action starts when they bind to specific receptors that are found on the target cell membrane (Le Roith and Blakesley, 2000). When a threshold amount of receptors becomes activated, the signal transduction process that ends up in a specific cellular activity is created (Vasita and Katti, 2006). Therefore, it can be concluded that effects of growth factors are both receptor concentration and time dependent (Croucher et al., 1999). Some of those growth factors are studied and characterized in detail and consequently, they are readily used as a part of

recombinant technology techniques and moreover, this enables a more articulated research of their potential in various tissue engineering applications.

Insulin-like growth factor 1 (IGF-1), a 70 amino acids polypeptide, is related to insulin structure and growth hormone regulates its synthesis. Besides, it is responsible for driving DNA synthesis in different cell types including chondrocytes, and also stands for most of the chondrocyte stimulating activity found in serum (Croucher et al. 1999; Clemmons, 2000). In case there is a mechanical stimulation found, insulin-like growth factor-I (IGF-I) can dramatically increase biosynthesis levels (Bonassar et al.2001; Jin et al. 2003). Additionally, increasing proteoglycan and collagen type II synthesis are among the positive effects of IGF-1, next to chondrocytes proliferation (Makower et al. 1989; Darling et al., 2005; Morales, 1997; Loeser et al., 2003; De Mattei et al. 2004). Furthermore, chondrocyte-based repair of osteochondral defects is suggested to be enhanced by IGF-1, *in vivo* (Fortier et al. 2002).

Transforming growth factor-β1 (TGF-β1) functions in enhancing matrix fabrication in engineered constructs, as well as stimulating cellular proliferation (Guerne et al. 1994; Darling and Athanasiou 2003; Blunk et al. 2002; van der Kraan et al.1992). This homodimeric, 25 kD protein is a member of a superfamily consisting of 100 related proteins. The bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDF) are the proteins found in this superfamily (Chin et al., 2004).

Synthesis of extracellular matrices is under the control of TGF- β1 and this control is exerted through the stimulation of the collagen, fibronectin (Ignotz et al., 1987) and proteoglycan (Chen et al., 1987; Morales, 1991) synthesis. Furthermore, TGF - β1 positively directs cartilage differentiation and repair (Boumediene et al., 1995; Johnstone et al., 1998; Grimaud et al., 2002; Olney et al., 2004; Darling et al., 2005). On the other hand, this factor is known for its specific negative side effects. Those undesired traits manifest, such as inflammatory responses and osteophyte formation in articular cartilage defects, if it is located in the knee joint too long (van Beuningen et al., 2000).

## 1.8. Controlled Delivery Systems

The term “controlled delivery” can be described as the administration of bioactive agents to control the in vivo location and concentration of these molecules for treatment of diseases (Uchegbu, 2006). Traditional methods of drug administration generally include pills, eye drops, ointments, and injections in veins as solutions, while novel drug delivery approaches have been started to be used nowadays. Those methods are carried out as drug modifications, usage of small particles for drug entrapment purposes and their insertion into the bloodstream, as well as enclosement of drugs within pumps or other polymeric materials and their replacement in the target body compartment.

In recent years, the interest in drug delivery has increased because of the increasing need of safe drugs that are able to target their destinations with minimum side effects. A perfect drug should reach only its target site, using the lowest concentration without negatively affecting the surrounding tissues that are out of target range. Current delivery systems use soluble, simple macromolecules (monoclonal antibodies, synthetic polymers, polysaccharides, biodegradable polymers), as well as complex multicomponent molecules (cells, cell ghosts, erythrocytes, lipoproteins, liposomes, microcapsules, microparticles) (Pierigé et al., 2008).

An ideal drug delivery system should be made self-powered and computer-controlled nanorobots, and be capable of precise timing and delivering of pharmaceuticals to the specific target in the body. Although such systems are not established yet, cell based delivery systems are the closest ones to the ideal drug delivery system: transduced cells and cell carriers. In the former case, cells are transduced by gene transfer method with selected genes that express fluorescent proteins in order to observe the cell’s behaviour *in vivo,* or to correct a genetic defect, or to make the target cell exposed to the action of a selected drug (i.e. by expressing Tymidine kinase). In the latter method, cells can be loaded with drugs that will be released in circulatory system, or to the target cells. Macrophages and erythrocytes have been reported as the most useful cell carriers for drug delivery (Pierigé et al., 2008).

Polymeric drug delivery systems are also widely used as drug delivery systems. Studies on these systems reported polymers that are capable of extending the

function of drug by entrapping the drug within matrices, converting drug release in the direction of tumors, direct therapeutic genes or oligonucleotides into cells, and triggering the release of drug only when there is a defined change in temperature or pH or when activated by an enzyme. Changing the water solubility of polymers, increasing their chain lengths via cross-linking them with copolymers and other groups, materials with enhanced drug delivery properties are produced, such as prolonged drug availability when formulated as hydrogels or microparticles (Uchegbu, 2006).

Biodegradable drug and growth factor carriers, such as polymer hydrogels and microparticles, collagen, fibrin, chitosan, and hyaluronan based materials, have been studied for cartilage tissue engineering. For instance, collagen sponges filled with BMP-2 (bone morphogenetic protein-2), and PLGA nanospheres loaded with betamethasone (a water-soluble corticosteroid) have been successfully applied for repairing of cartilage defects (Holland and Mikos, 2003).

### 1.9 Aim

The objective of this thesis is the investigation of pH and temperature sensitive nanospheres as a tool for growth factor delivery systems on PLGA porous scaffolds for cartilage tissue engineering.

# 2. MATERIALS AND METHODS

## 2.1. Materials

### 2.1.1. Isolation and Growth of Mesenchymal Stem Cells and Articular Cartilage Cells

#### 2.1.1.1. Isolation of Mesenchymal Stem Cells From Bone Marrow

- Dulbecco's Modified Eagle Medium (DMEM; low glucose) (Gibco – Invitrogen, USA)
- Dulbecco's Modified Eagle Medium (DMEM; high glucose) (Sigma-Aldrich Corporation, Germany)
- Fetal Bovine Serum (FBS) (Gibco – Invitrogen, USA)
- Penicilin/Streptomycin Solution (Biochrom Ac, Germany)
- T75 Tissue Culture Flasks (Orange Scientific, Belgium)
- Petri dishes (ISOTHERM, Turkey)
- Sterile syringes (Set®Inject, Turkey)

#### 2.1.1.2. Mesenchymal Stem Cell Culturing

- Phosphate Buffered Saline (D-PBS, 500 mM, pH 7.4) (Gibco – Invitrogen, USA)
- Dulbecco's Modified Eagle Medium (DMEM; high glucose) (Sigma-Aldrich Corporation, Germany)
- Fetal Bovine Serum (FBS) (Gibco – Invitrogen, USA)
- Penicilin/Streptomycin Solution (Biochrom Ac, Germany)

#### 2.1.1.3. Trypsinization and Subculturing of Mesenchymal Stem Cells

- Trypsin/EDTA (Gibco – Invitrogen, USA)
- Fetal Bovine Serum (FBS) (Gibco – Invitrogen, USA)
- Dulbecco's Modified Eagle Medium (DMEM; high glucose) (Sigma-Aldrich Corporation, Germany)
- Propidium Iodide (PI) (INCYTO, Korea)

- C-Reader Automatic Cell Counter Reagents (INCYTO, Korea)
- C-Reader Chip (INCYTO, Korea)
- T75 Tissue Culture Flasks (Orange Scientific, Belgium)

### 2.1.2. Preparation and *in vitro* Characterization of Nanospheres

#### 2.1.2.1. Preparation of Growth Factor Loaded PLGA Nanospheres

- Dichloromethane (DCM) (AppliChem, Germany)
- Poly(lactic acid-co-glycolic acid) (PLGA, 50:50) (Boehringer-Ingelheim, Germany)
- Ultrasonic Homogenizer (4710 series, Cole-Parmer Instruments, USA)
- Polyvinyl Alcohol (PVA) (Fluka, Switzerland)
- Tris (AppliChem, Germany)
- Hydrochloric Acid (HCl) (Riedel-de Haen, Germany)

#### 2.1.2.2. Preparation of Growth Factor Loaded NIPAM Nanospheres

- MBA crosslinked poly(NIPAM)
- Acetone (AppliChem, Germany)

#### 2.1.2.3. *In vitro* Characterization of Growth Factor Loaded Nanospheres

- Dulbecco's Modified Eagle Medium (DMEM; high glucose) (Sigma-Aldrich Corporation, Germany)
- Penicilin/Streptomycin Solution (Biochrom Ac, Germany)
- Insulin – Transferrin – Selenium (ITS, GIBCO – Invitrogen, USA)
- Linoleic acid (Sigma-Aldrich Corporation, Germany)
- L-Proline (Sigma-Aldrich Corporation, Germany)
- Sodium-pyruvate (Sigma-Aldrich Corporation, Germany)
- Albumin, Bovine Fraction V (Sigma-Aldrich Corporation, Germany)
- L-ascorbic acid (AppliChem, Germany)
- Dexamethasone (AppliChem, Germany)
- Empty PLGA nanospheres (EP)
- Empty NIPAM nanospheres (EN)
- IGF-I loaded PLGA nanospheres (IP)
- TGF-β1 loaded NIPAM nanospheres (TN)

### 2.1.3. Preparation and *in vitro* Characterization of Nanospheres on PLGA Scaffolds

#### 2.1.3.1. Porous PLGA Scaffold Preparation

- Poly(lactic acid-co-glycolic acid) (PLGA, 50:50) (Boehringer-Ingelheim, Germany)
- Dichloromethane (DCM, AppliChem, Germany)
- NaCl crystals (300 μm–500 μm) (Sigma-Aldrich Corporation, Germany)
- Glass Petri Dishes (ISOTHERM, Turkey)
- Distilled water
- Freeze-dryer (Thermo, Savant Modulyo Freeze Drying Systems, USA)

#### 2.1.3.2. Scanning Electron Microscopy of Empty Porous PLGA Scaffolds

- Sputter Coater (Bal-tec SCD 005, Germany)
- Scanning Electron Microscope (Carl Zeiss EVO, Germany)

#### 2.1.3.3. Preparation of Cell Containing Porous PLGA Scaffolds

- Ethanol, absolute (J.T.Baker, Holland)
- Dulbecco's Modified Eagle Medium (DMEM; low glucose) (Gibco – Invitrogen, USA)
- Chondroitin Sulfate A (Sigma-Aldrich Corporation, Germany)
- Vacuum system (BioVac225 Suction Systems)
- Nanospheres (EP, EN, IP, TN)
- C-reader Automatic Cell Counter (INCYTO, CRM-350)
- Alginic acid (Sigma-Aldrich Corporation, Germany)
- Phosphate Buffered Saline (D-PBS, 500 mM, pH 7.4) (Gibco – Invitrogen, USA)
- 24-well plates (Orange Scientific, Belgium)

#### 2.1.3.4. Cell Growth on Cell Containing Porous PLGA Scaffolds

- CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, USA)
- MTS Cell Proliferation Assay Solution (Promega, USA)
- Dulbecco's Modified Eagle Medium (DMEM; low glucose) (Gibco – Invitrogen, USA)
- 96-well plates (Orange Scientific, Belgium)

- Elisa Plate Reader (Bio-Tek, Elx800, USA)

#### 2.1.3.5. Scanning Electron Microscopy of Cell Containing Porous PLGA Scaffolds

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

#### 2.1.3.6. Confocal Microscopy with Collagen Type II and Aggrecan Double Staining of Cell Containing Porous PLGA Scaffolds

- Formaldehyde (Fluka, Switzerland)
- Tween® 20 (AppliChem, Germany)
- Methanol (J.T.Baker, Holland)
- Phosphate Buffered Saline (D-PBS, 500 mM, pH 7.4) (Gibco – Invitrogen, USA)
- Fetal Bovine Serum (FBS) (Gibco – Invitrogen, USA)
- Collagen Type II, mouse monoclonal $IgG_{2b}$ (Santa Cruz Biotechnology, Inc., USA)
- Aggrecan, rabbit polyclonal IgG (Santa Cruz Biotechnology, Inc., USA)
- Goat anti-mouse IgG – FITC (Santa Cruz Biotechnology, Inc., USA)
- Alexa Fluor ® 647, goat anti-rabbit IgG (Invitrogen., USA)
- Mowiol (Calbiochem, Germany)

## 2.2. Methods

There are two sets of experiments in this study: 1- the effect of nanospheres on cells seeded into 24-well plate tissue culture polystyrene (TCPS), 2- the effect of the same nanospheres on cells seeded into 3D scaffolds. In the first set, different mediums containing different nanospheres were added onto the cells. In the second set, the same procedure was applied to the cells seeded on scaffolds (Figure 2.1)

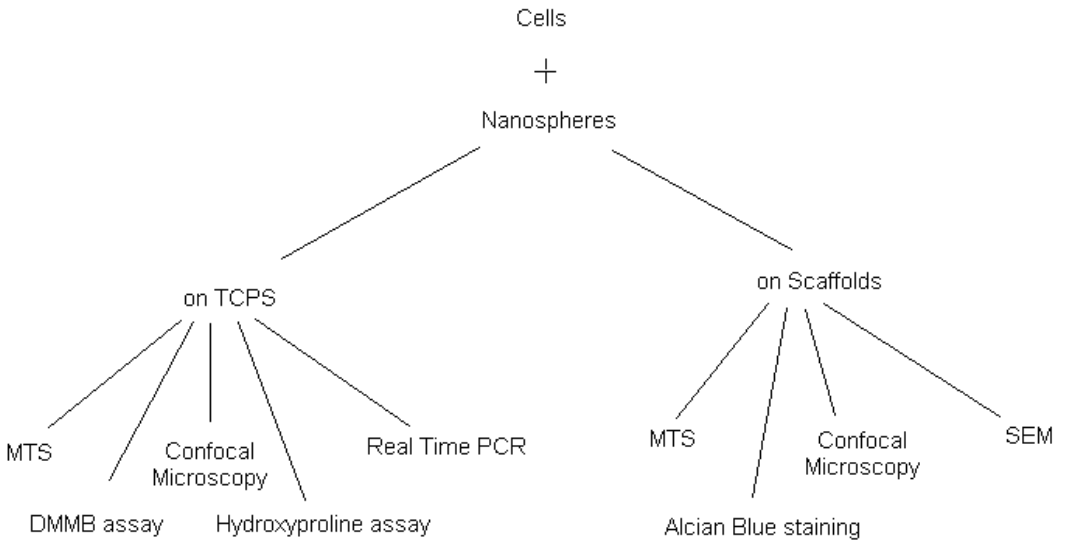


**Figure 2.1.** Scheme of the experimental pathway.

### 2.2.1. Isolation and Growth of Mesenchymal Stem Cells and Articular Cartilage Cells

#### 2.2.1.1. Isolation of Mesenchymal Stem Cells From Bone Marrow

6 week old, 150-170g male Sprague-Dawley rats' bone marrow stromal osteoblastic cells were obtained for isolation. After clearing the femur, Dulbecco's Modified Eagle Medium including 1000 unit/mL streptomycin was used to wash the bones. The two extremes of the femur were cut and the marrow was placed into petri dishes containing 5 mL growth medium (DMEM with %20 fetal bovine serum (FBS) and 100 unit/mL penicillin and 100 unit/mL streptomycin) by using syringe. The cell groups were separated by continuous pipetting method and they were centrifuged at 2000 rpm for 5 min. The pellet was dissolved in 12 mL primer culture medium and cultivated in T-75 flask. The cells were incubated in $CO_2$ incubator at 37°C, in 5% $CO_2$ and 90% humidity ambient.

#### 2.2.1.2. Mesenchymal Stem Cell Culturing

The hematopoietic stem cells and other unhold cells were excluded from the flasks by washing with PBS (500 mM, pH 7.4). DMEM-high glucose with 10%FBS and 100 unit/mL penicillin-streptomycin was added on cells. The cells were incubated at 37°C, in 5% $CO_2$ and 90% humidity ambient and the medium was refreshed every other day.

#### 2.2.1.3. Trypsinization and Subculturing of Mesenchymal Stem Cells

At the end of 7 to 10 days of incubation, cells were detached with Trypsin (0.25%)-EDTA treatment for 5 min at 37°C. After detachment, trypsin was deactivated with serum and the cells were collected by centrifugation at 2300 rpm for 5 min. Pellet was resuspended in growth medium. Then, total cell number was counted with C-reader automatic cell counter. Propidium iodide (PI) provided the DNA staining and C-Reader Chip two channel hemocytometer chips were used for the counting. The cells were transferred into two 75 $cm^2$ flasks. The subcultures were incubated at 37°C, in 5% $CO_2$ and 90% humidity ambient and the medium was refreshed every other day.

#### 2.2.1.4. Determination of Mesenchymal Stem Cell Number

To determine the viable mesenchymal cell number, C-reader automatic cell counter was used. Propidium iodide (PI) provided the DNA staining and C-Reader Chip two channel hemocytometer chips were used for the counting. One hundred µl cell containing medium mixed with total cell counting solution and non-viable cell counting solution.

#### 2.2.1.5. Isolation and Growth of Cartilage Cells From Articular Cartilage

The Sprague-Dawley rats' knee joint cartilage was isolated under sterile conditions. RPMI 1640 with 1000 unit/mL penicillin-streptomycin was used to wash the samples. The cartilage tissue samples that were taken into 35 mm petri plates were cut into small pieces around 0.5 mm by using scalpel. After washing and cleaning the samples with RPMI 1640 medium, they were incubated overnight in 2 mg/mL type-II collagenase solution for enzymatic cell separation. At the end of 48 hours of incubation, the cells were pipetted and transferred into a new tube.

RPMI 1640 (3 mL) medium was added onto the cells and they were centrifuged for 5 min. Then, 10 mL culture solution was added onto the cells in the pellet and they were homogenized by glass pipette. The sample was resuspended and separated in flasks. Three mL RPMI 1640 medium (100 mL, 10% mL FBS, 1.5 mL L-glutamine, 1 mL penicillin-streptomycin) was added onto each flask and the primer cells were incubated at 37°C', in %5 $CO_2$ and %90 humidity ambient. The adhesion of the cells on the flask surface and the growth of cells were visualized by inverted microscope (Nikon, Eclipse, TS100, Japan). The medium was refreshed every other day.

#### 2.2.1.6. Articular Cartilage Cell Culturing

The hematopoietic stem cells and other unhold cells were excluded from the flasks by washing with PBS (500 mM, pH 7.4). DMEM-high glucose with 10%FBS and 100 unit/mL penicillin-streptomycin was added on cells. The cells were incubated at 37°C, in 5% $CO_2$ and 90% humidity ambient and the medium was refreshed every two days.

#### 2.2.1.7. Trypsinization and Subculturing of Articular Cartilage Cells

At the end of 7 to 10 days of incubation, cells were detached with Trypsin (0.25%)-EDTA treatment for 5 min at 37°C. After detachment, trypsin was deactivated with serum and the cells were collected by centrifugation at 2300 rpm for 5 min. Pellet was resuspended in growth medium. Then, total cell number was counted and the cells were transfered into two 75 cm² flasks. The subcultures were incubated at 37°C, in 5% $CO_2$ and 90% humidity ambient and the medium was refreshed every other day.

#### 2.2.1.8. Determination of Articular Cartilage Cell Number

To determine the viable Articular Cartilage cell number, C-reader automatic cell counter was used. Propidium iodide (PI) provided the DNA staining and C-Reader Chip two channel hemocytometer chips were used for the counting. One hundred µl cell containing medium mixed with total cell counting solution and non-viable cell counting solution.

### 2.2.2. Preparation and *in vitro* Characterization of Nanospheres

#### 2.2.2.1. Preparation of Growth Factor Loaded PLGA Nanospheres

Nanospheres encapsulating IGF-I were prepared by the double emulsion-solvent evaporation technique by Pınar Yılgör (METU, BIOMAT Research Center, Biotechnology Dept). Briefly, the agent was disolved in dichloromethane (DCM) containing PLGA by probe sonication for 15 s (Ultrasonic homogenizer, 4710 series, Cole-Parmer Instruments, USA) at an output of 50 W. The first emulsion ($w_1/o$) was added into an aqueous solution of PVA (4% (w/v)) to form the second emulsion by sonication ($w_1/o/w_2$). The double emulsion was then added into PVA (0.3% (w/v)) and the organic solvent was evaporated by vigorous stirring overnight. Nanospheres were collected by centrifugation (15000 g, 10 min) and washed twice with Tris-HCl

(pH 7.4). The nanospheres were then resuspended in the buffer and lyophilized (-80$^{o}$C, 6.10$^{-2}$ mbar).

#### 2.2.2.2. Preparation of Growth Factor Loaded NIPAM Nanospheres

Nanoparticles were prepared by nanoprecipitation method by Banu Bayyurt (METU, BIOMAT Research Center, Biotechnology Dept). MBA crosslinked poly(NIPAM) (0.04%, v/v) were precipitated from organic solvent, acetone, into cyclohexane without using any surfactant. Model protein (BSA) or growth factor (TGF-β1) loading was accomplished by equilibrium partitioning method. In a typical loading study, the solution of agent (2 mg) was added to the nanoparticle dispersion (50 mg/mL in PB solution) to form the nanoparticle suspension. The suspension was cooled to 4 °C, stored for 24 h, and heated quickly to 37 °C. Nanoparticle suspension containing free agent was centrifuged at 13 500 rpm, 20 min and after removing supernatant, nanoparticles were dried at 37 $^{o}$C in the oven, overnight.

#### 2.2.2.3. *In vitro* Characterization of Growth Factor Loaded Nanospheres

For *in vitro* characterization of nanospheres, different medium contents were prepared (Table 2.1). For that purpose, cartilage differentiation medium (DMEM-high glucose with 100 unit/mL penicillin-streptomycin, 6.25 µg/mL insulin, 6.25 µg/mL transferrin, 6.25 µg/mL selenic acid, 5.33 µg/mL linoleic acid, 40 µg/mL proline, 100 µg Na-pyruvate, 1.25 mg/mL BSA, 50 µg/mL L-ascorbic acid ve 100 nM dexamethasone) was put into 24 well plates containing cells. Then, empty PLGA nanospheres (EP), empty NIPAM nanospheres (EN), IGF loaded PLGA nanospheres (IP), TGF-β1 loaded NIPAM nanospheres (TN), IGF loaded PLGA nanospheres and IGF loaded NIPAM nanospheres both (IPTN) were added onto each well, respectively. Each combination was prepared three times.

**Table 2.1.** Medium contents of cell seeded samples in 24 well plate.

| Sample | PLGA nanosphere | NIPAM nanosphere | Cell |
|---|---|---|---|
| **Sample 1 (OC)** | | | **X** |
| **Sample 2 (EP)** | **X (empty)** | | **X** |
| **Sample 3 (EN)** | | **X (empty)** | **X** |
| **Sample 4 (IP)** | **X (IGF-1)** | | **X** |
| **Sample 5 (TN)** | | **X(TGF-β1)** | **X** |
| **Sample 6 (IPTN)** | **X (IGF-1)** | **X (TGF-β1)** | **X** |

#### 2.2.2.4. Determination of Cell Growth - Calibration Curve

A calibration curve of cell numbers vs absorbance was constructed. Certain numbers of cells were seeded onto 24 well plate wells and waited for two hours. CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was used to determine the cell density. DMEM low glucose medium was mixed with MTS one solution with a ratio 5:1. Cells were washed with PBS to remove artifacts. 500 μL of MTS- medium mix was added onto each sample in the 24-well plate and incubated for 4 h at 37ºC in a $CO_2$ incubator. After 4 h of incubation, 200 μL of solution from each well was transferred into a new 96-well plate in triplicate. Absorbance was determined at 490 nm using an Elisa Plate Reader (Bio-Tek, Elx800, USA).

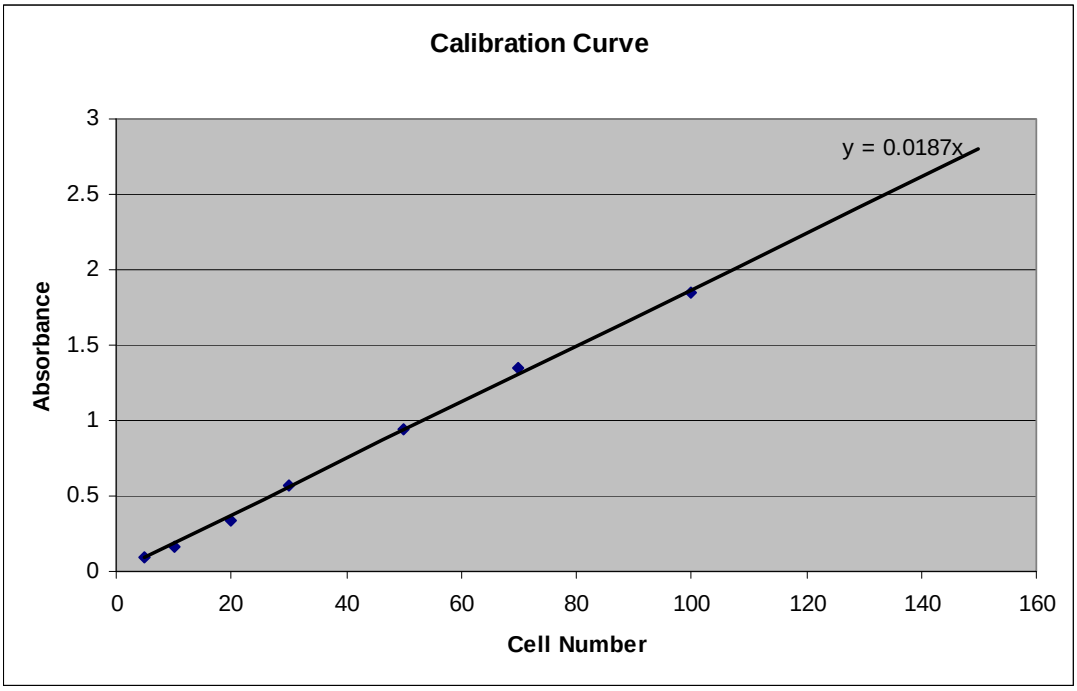


**Figure 2.2** Calibration curve of bone marrow mesenchymal stem cells of rat with MTS for 4 hours.

### 2.2.2.5. Determination of Cell Growth in Nanosphere Containing Medium

In order to determine the cell growth in nanosphere containing medium, 24 well plates with cells and different medium compositions were prepared as it was described in section 2.2.2.3. Each cell and medium composition was prepared in triplicate for days Days 1, 7 and 14. At the end of the time periods, MTS assay was carried out onto each sample as it was described in section 2.2.2.4. A graph of Cell Numbers vs Absorbance was constructed. Cell numbers were calculated according to the calibration curve constucted before.

### 2.2.2.6. RT-PCR

Total RNAs were isolated from the cells in different medium composition (described in section 2.2.2.3) by using RNeasy Mini Kit. First-strand cDNA synthesis was performed by using Sensiscript Reverse Transcriptase. Primer sets for each gene are listed in Table 2.2. For optimization of the primers, the amplified DNA fragments at different tempratures were visualized through 1.5% agarose gel electrophoresis, stained with ethidium bromide and photographed under UV light. In real-time PCR experiments for each sample 12,5 µl SYBR Premix Ex Taq (2X), 0,5

µl PCR forward primer, 0,5 µl PCR reverse primer, 2 µl template, 9 µl $dH_2O$ were used in Bio-Rad iCycler™ real time system.

**Table 2.2.** Primers for PCR

| Marker | Sequence | Length |
|---|---|---|
| Beta actin | forward 5' TTCTACAATGAGCTGCGTGTG 3' | 125 bp |
| | reverse 5' GCTGGGGTGTTGAAGGTC 3' | |
| Collagen type II | forward 5' TGAACAACCAGATCGAGAGCA 3' | 175 bp |
| | reverse 5' CCAGTCTCCATGTTGCAGAAG 3' | |

### 2.2.2.7. Determination of ECM Storage in Cell Culture by Biochemical Methods- DMMB (1,9-dimethylmethylene blue) Assay

Determination of ECM storage in cell culture was carrried out with the cells in different medium composition (described in section 2.2.2.3) by using DMMB assay. Cells seeded on polypropylene tubes. At the end of each time period, samples were digested in 300 µg/mL of papain in 20 mM sodium phosphate (pH 6.8)**,** 1 mM EDTA and 2 mM dithiothreitol at 60°C for 1 hour. Sixteen mg/L DMMB solution was preparared with glycine, NaCl and HCl and final pH was adjusted to 3. Digested samples (100 µL) were mixed with 200 µL of DMMB solution and the absorbance was measured at 525nm using Thermo LabSystems Multiscan Spectrum (Model no1500, USA) microplate reader.

### 2.2.2.8. Determination of ECM Storage in Cell Culture by Biochemical Methods-Hydroxyproline Assay

Fifty µL of the different set of the same samples described in section 2.2.2.7 were hydrolyzed with 50 µL of 12M HCl for 18 h at 100 °C. The hydrolysate was then, dried in a vacuum desicator over the NaOH pellets. The residue was dissolved in 150 µL water, transferred into a 96-well plate, and dried in a hood. Sixty µL of water was then added onto each well followed by addition of 20 µL of assay buffer (1-propanol/water/pH 6 buffer*; 3:2:10 ratio). The solution was allowed to shake until the residue was fully solubilized. Then, chloramine T reagent (40µL, 0.050 mol/L), was added onto each sample and they were allowed to shake for 15 min at room temperature. DMBA reagent (80 µL dimethylaminobenzaldehyde (2 g), 1.25 mL of 1-propanol, 2.75 mL of perchloric acid) was added and the plate was incubated on a shaker for 20 min at 70 °C. After the plate was allowed to cool, absorbances were determined at 570 nm by using Thermo LabSystems Multiscan Spectrum (Model

no1500, USA) microplate reader. Data were converted to nanograms of hydroxyproline based on a standard curve that consists of hydroxyproline levels ranging from 20–2000 ng/well (Pratta et al., 2003).

pH 6 buffer*: (0.24 M citric acid, 0.88 M anhydrous sodium acetate trihydrate, 0.88 M anhydrous sodium acetate, 0.21 M acetic acid, 0.85 M sodium hydroxide).

#### 2.2.2.9. Determination of Cell Morphology in Cell Culture by Immunohistochemical Methods-Alexa Fluor® 546 Phalloidin and TO-PRO®-3 Confocal Microscopy Staining

Cell morphology in cell culture was also carrried out with the cells in different medium composition (described in section 2.2.2.3) by using Alexa Fluor® 546 Phalloidin and TO-PRO®-3 Confocal Microscopy Staining.

First, the samples were washed with PBS and incubated in 3% FBS/PBS for 10 min. Phalloidin was prepared in FBS/PBS (1.5%, 1:100). The serum was extracted from the samples by pipetting and antibody (Ab) solution was added onto each samples. The samples were incubated at the room temperature for 20 min to 1 h. After the incubation, the samples were washed with PBS for three times and every time they were waited for 5 min.. TO-PRO®-3 was prepared in 2% FBS/PBS (1:100). After the addition of TO-PRO®-3, the samples were incubated at the room temperature for 15 min. After the incubation, the samples were washed with PBS for three times and every time they were waited for 5 min.. They were mounted by 80- 100 µL mounting medium. The images were obtained with a Leica TCS SP2 Laser Scanning Spectral Confocal System (Leica microsystems GmbH, Heidelberg, Germany).

#### 2.2.2.10. Determination of ECM Deposition in Cell Culture by Immunohistochemical Methods-Collagen Type II and Aggrecan Confocal Microscopy Double Staining

Another way to determine cartilage marker proteins in cell culture with different medium composition (described in section 2.2.2.3) is the double staining of samples with collagen type II and aggrecan and observe them under the Confocal Microscopy.

In this method, the samples were washed with PBS for three times and waited in 3% FBS/PBS for 10 min. Collagen Type II primer Ab was prepared in 1.5% FBS/PBS (1:100). The serum was extracted from the samples by pipetting and Ab solution was added onto each samples before washing step. The primers were incubated at room

temperature for 1-18 h. After the incubation, the samples were washed with PBS for three times and every time the samples were waited for 5 min. Collagen type II secondary Ab (FITC) were prepared in 1% FBS/PBS (1:200). After the washing steps, secondary Ab solution was added onto each samples and they were incubated at $37^0$C for 45 min. After the incubation, the samples were washed with PBS for three times and every time they were waited for 5 min. Another primary antibody, aggrecan, was prepared in 1.5% FBS/PBS (1:200). After the washing steps, Aggrecan antibody solution was added onto each samples and they were incubated at the room temperature for 1-18 h. After the incubation, the samples were washed with PBS for three times and every time the samples were waited for 5 min. Secondary aggrecan antibody was prepared in 1% FBS/PBS (1:200). After the washing steps, Secondary aggrecan Ab solution was added onto each samples and they were incubated at $37^0$C for 45 min. After the incubation, the samples were washed with PBS for three times and they were mounted by 80-100 µL mounting medium. The images were obtained with a Leica TCS SP2 Laser Scanning Spectral Confocal System (Leica microsystems GmbH, Heidelberg, Germany).

### 2.2.3. Preparation and *in vitro* Characterization of Nanospheres on PLGA Scaffolds

#### 2.2.3.1. Porous PLGA Scaffold Preparation

PLGA Scaffolds (8%, 50:50) were prepared in DCM. In order to obtain porous structure, NaCl crystals (300 µm–500 µm) were used. The polymer solution was poured into the glass. Petri plates that were covered with aluminum foil. Plates were left in fume hood for DCM evaporation. For the following two days, scaffolds were placed in distilled water on a magnetic stirrer in order to remove salt particles from the sponges. The scaffolds were removed from the water, freezed at -20 $^o$C and lyophilized using a freeze-dryer (Thermo, Savant Modulyo Freeze Drying Systems, USA). After lyophilization, the scaffolds were cut to be 13 mm in diameter and then placed in 24-well-plates.

#### 2.2.3.2. SEM of Empty Porous PLGA Scaffolds

Scaffolds were covered by sputter coater (Bal-tec SCD 005) with 5nm gold. To obtain scanning electron microscope (SEM) images, samples were viewed using a Carl Zeiss EVO 40 Scanning Electron Microscope operated at 10.00 kV accelerating.

### 2.2.3.3. Determination of Porosity of Empty Porous PLGA Scaffolds

Average pore size and the pore distribution of the porous PLGA scaffolds were examined using the Scion Image Analysis Program (National Institudes of Health (NIH) image) which is a public domain image processing and analysis program for the Macintosh.

### 2.2.3.4. Degradation Study of Empty Porous PLGA Scaffolds

For the degradation study, PLGA scaffolds were weighed. The disks were put into 50 mL Corning flasks containing phosphate buffer (20 mL, pH 7.4, 10 mM, 0.09% sodium azide) and incubated at 37 $^{o}$C for 1, 15, 30, 60 and 120 days. Samples of solution from each foam were collected at different time points. At the end of the period, the disks were freeze-dried for 8 h and weighed. At the same time, the pH in the supernatant (phosphate buffer) was recorded. All measurements were expressed as means ± standard deviation (sd) relative to the initial values. This experiment was performed in dublicate.

### 2.2.3.5. Preparation of Cell Containing Porous PLGA Scaffolds

Porous PLGA Scaffolds were prepared as described in section 2.2.3.1. They were put into 24 well plates and 500 µL of 70% ice-cold ethanol was added onto each well. The plates were kept in 4$^{0}$C for 1 h for sterilization. Then, the plates were washed with cell culture media to remove ethanol. At the end of washing, the scaffolds were air dried under the laminar flow cabinet. Chondroitin sulfate (CS) (250 µL of 1%) was added onto each well and its saturation in the scaffolds was obtained by vacuum (BioVac225 Suction Systems) at 40 cm/Hg, 30 seconds for three times. After removing the excess solution from the wells, vacuum was applied for 30 seconds once more.

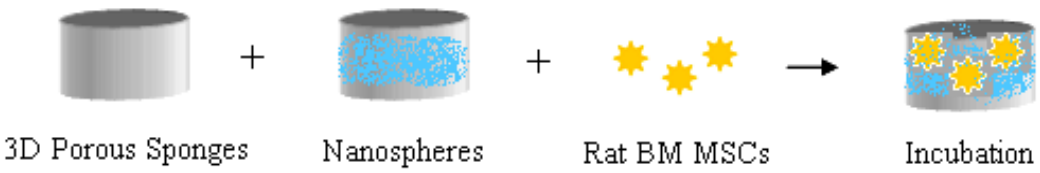


**Figure 2.3.** Illustration of cell seeding onto porous PLGA sponges.

Then, different nanosphere combinations such as empty PLGA nanospheres (EP), empty NIPAM nanospheres (EN), IGF loaded PLGA nanospheres (IP), TGF-β1

loaded NIPAM nanospheres (TN), IGF loaded PLGA nanospheres and IGF loaded NIPAM nanospheres both (IPTN) were added onto the PLGA scaffolds as presented in Table 2.3. After the application of vacuum for 30 seconds, seven different samples (with and without chondroitin sulphate controls and five different nanosphere content) were ready for cell seeding.

**Table. 2.3.** Cell seeded PLGA Sponges with different contents.

| Sample | PLGA scaffold | Chondroitin Sulphate | PLGA nanosphere | NIPAM nanosphere | Cell | Alginate |
|---|---|---|---|---|---|---|
| Sample 1 (OC) | X | | | | X | |
| Sample 2 (CHA) | X | X | | | X | X |
| Sample 3 (EP) | X | X | X (empty) | | X | X |
| Sample 5 (EN) | X | X | | X (empty) | X | X |
| Sample 4 (IP) | X | X | (IGF-1) | | X | X |
| Sample 6 (TN) | X | X | | (TGF-β1) | X | X |
| Sample 7 (IPTN) | X | X | IGF-1 | TGF-β1 | X | X |

In the next step, the cells were detached from the culture dishes and counted with C-reader automatic cell counter (INCYTO, CRM-350). Each scaffold was seeded with 25.000 cells/cm$^2$ and vacuum was applied for 30 seconds again. Afterwards, the plates were incubated for 2 h at 37$^0$C, 5% $CO_2$. When the cells attached to the PLGA scaffolds, 2% alginic acid (500 µL) was added onto each well and vacuum was applied for 30 seconds for once. The scaffolds were washed with PBS once and 1 mL cartilage differentiation medium (DMEM-high glucose with 100 unit/mL penicillin-streptomycin, 6.25 µg/mL insulin, 6.25 µg/mL transferrin, 6.25 µg/mL selenic acid, 5.33 µg/mL linoleic acid, 40 µg/mL proline, 100 µg Na-pyruvate, 1.25 mg/mL BSA, 50 µg/mL L-ascorbic acid ve 100 nM dexamethasone) was added onto each well and then the plates were incubated into $CO_2$ incubator at 37$^0$C.

### 2.2.3.6. Cell Growth on Cell Containing Porous PLGA Scaffolds

CellTiter 96® AQueous One Solution Cell Proliferation Assay was used to determine the cell density inside the polymer scaffolds. The experiment was performed in triplicate at days 1, 7 and 14 after seeding the scaffolds with cells. DMEM low glucose medium was mixed with MTS one solution with a ratio 5:1. Cell seeded PLGA scaffolds were transferred into a new, sterile 24-well plate and washed with PBS in order to remove any left over medium. MTS- medium mix (500 µL) was

added onto each sample in 24-well plate and incubated for 4 h at 37ºC in a $CO_2$ incubator. After 4 h of incubation, 200 µL of solution from each well was transferred into a new 96-well plate in triplicate. Absorbance was determined at 490 nm using an Elisa Plate Reader (Bio-Tek, Elx800, USA).

#### 2.2.3.7. SEM of Cell Containing Porous PLGA Scaffolds

At the end of 1, 7 and 14 days of incubation, SEM samples were washed with cacodylate buffer for three times and to fix the cells they waited in 2,5% gluteraldehyde for one hour and washed with Cacodylate Buffer (0.1 M, pH 7.4) for three times. They were washed and refrigerated in 0.1 M of sodium cacodylate buffer (pH 7.4) until processed for SEM. Before use, they were rinsed with deionized water and stored in a deep freezer (-80 $^{o}$C). Before SEM observation, samples were freeze-dried for 8h and coated with gold by sputter coater (Bal-tec SCD 005). SEM was carried out in a Carl Zeiss EVO 40 Scanning Electron Microscope operated at 10.00 kV accelerating.

#### 2.2.3.8. Confocal Microscopy with Collagen Type II and Aggrecan Double Staining of Cell Containing Porous PLGA Scaffolds

The samples were prepared as it was described in section 2.2.3.6. At the end of 1, 7 and 14 days of incubation, they were washed with PBS for three times and waited in 3% FBS/PBS for 10min. Collagen Type II primary Ab was prepared in 1.5% FBS/PBS (1:100). The serum was extracted from the samples by pipetting and Ab solution was added onto each samples before washing step. The primers were incubated at the room temperature for 1-18 h. After the incubation, the samples were washed with PBS for three times and every time the samples were waited for 5 min. Collagen type II secondary Ab (FITC) was prepared in 1% FBS/PBS (1:200). After washing steps, secondary Ab solution was added onto each samples and they were incubated at $37^{0}$C for 45 min. After the incubation, the samples were washed with PBS for three times and every time the samples were waited for 5 min. Another primary Ab, Aggrecan, was prepared in 1.5% FBS/PBS (1:200). After the washing steps, Aggrecan Ab solution was added onto each sample and they were incubated at the room temperature for 1-18 h. After the incubation, the samples were washed with PBS for three times and every time the samples were waited for 5 min. Secondary Aggrecan Ab was prepared in 1% FBS/PBS (1:200). After the washing steps, secondary aggrecan Ab solution was added onto each sample and they were

incubated at $37^0$C for 45 min. After the incubation, the samples were washed with PBS for three times and they were mounted by 80-100 μL mounting medium.

### 2.2.3.9. sGAG Production on Cell Containing Porous PLGA Scaffolds

sGAG production of the cells in the matrix were analysed by Alcian Blue staining kit (pH 2,5). Samples (described in section 2.2.3.6) were washed with $dH_2O$ and 10 drops of reagent A was put onto them. After waiting for 30 minutes, the solution was drained and 10 drops of reagent B was added onto them. At the end of 10 min. of incubation, the samples were washed with $dH_2O$ and 10 drops of reagent C were added. After washing with $dH_2O$, samples were examined under inverted microscope.

# 3. RESULTS

## 3.1. Isolation of MSCs from Bone Marrow and Articular Cartilage Cells

MSCs from bone marrow of the rat femur and tibia and articular cartilage cells from rat knee joint cartilage were isolated as described in sections 2.2.1.1 and 2.2.1.5, respectively. Their morphologies were determined under the light microscope. Fibroblastic morphologies of the cells were observed (Figure 3.1).

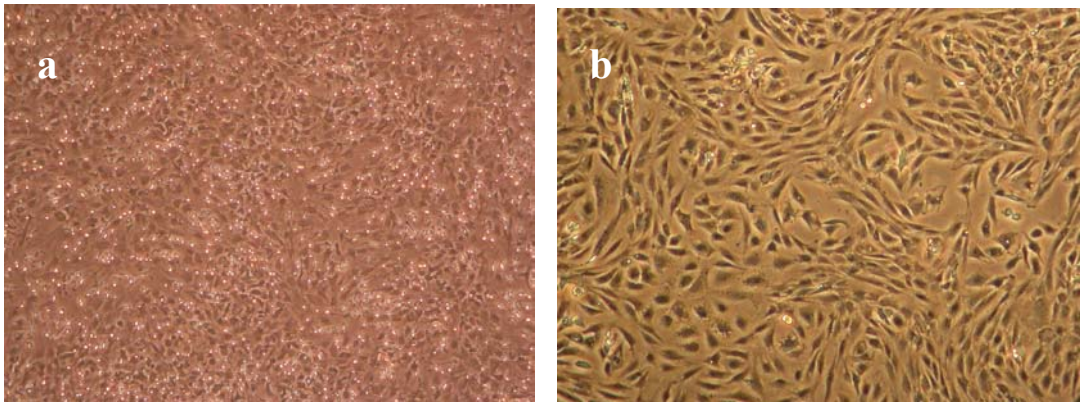


**Figure 3.1.** Monolayer growth of (a) bone marrow derived MSCs and (b) articular cartilage cells (x4).

## 3.2. *In vitro* Characterization of Growth Factor Loaded Nanospheres

### 3.2.1. SEM

The surface characteristics of pH sensitive PLGA and temperature sensitive NIPAM nanospheres were determined by SEM.

SEM micrographs show that both pH sensitive PLGA nanospheres and temperature sensitive NIPAM nanoparticles were less than 1 μm in diameter and had spheroid shapes (Figure 3.2).

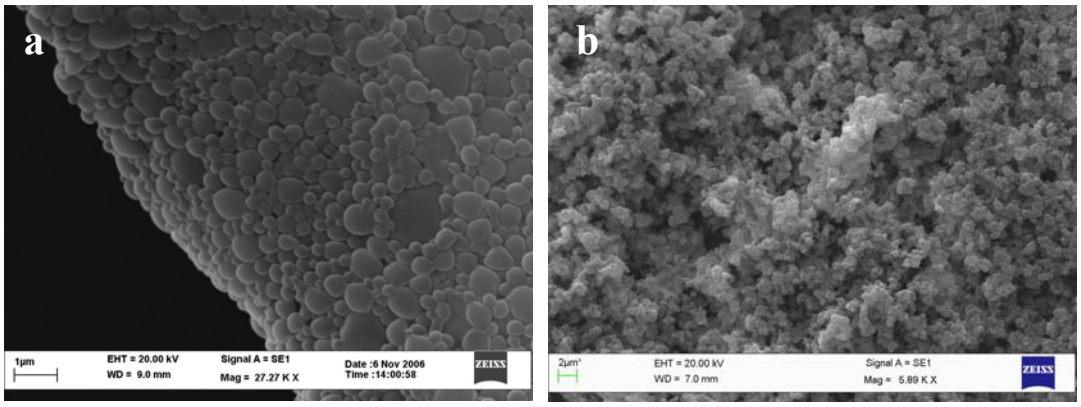


**Figure 3.2.** SEM of (a) pH sensitive PLGA nanospheres and (b) temperature sensitive NIPAM nanospheres.

### 3.2.2. Determination of Cell Growth - Calibration Curve

The calibration curve is presented in Appendix - A1.

### 3.2.3. Determination of Cell Growth in Nanosphere Containing Medium

At the end of 1, 7 and 14 days of incubation in different culture medium (Table 3.1) proliferation of bone marrow derived mesenchymal stem cells were measured by MTS assay.

**Table 3.1.** Abbreviations of the different culture mediums.

| Abbreviation | Culture Medium |
|---|---|
| OC | only differentiation medium |
| EP | differentiation medium with empty PLGA nanospheres |
| EN | differentiation medium with empty NIPAM nanospheres |
| IP | differentiation medium with IGF-I loaded PLGA nanospheres |
| TN | differentiation medium with TGF-β1 loaded NIPAM nanospheres |
| IPTN | differentiation medium with IGF-I loaded PLGA nanospheres + TGF-β1 loaded NIPAM nanospheres |

Initial cell number was 10.000 cells/well for the cells on TCPS. The cell numbers in OC increased throughout the 14 days of incubation (Figure 3.4). Cells with IP, TN, and IPTN showed similar growth patterns with OC. However, the growth rate of cells with EP and EN (50.000 and 68.000 cells/well, respectively) decreased drastically after 14 days in culture. Cell growth in IPTN was found to be between IP and TN in 7 and 14 days of incubation.

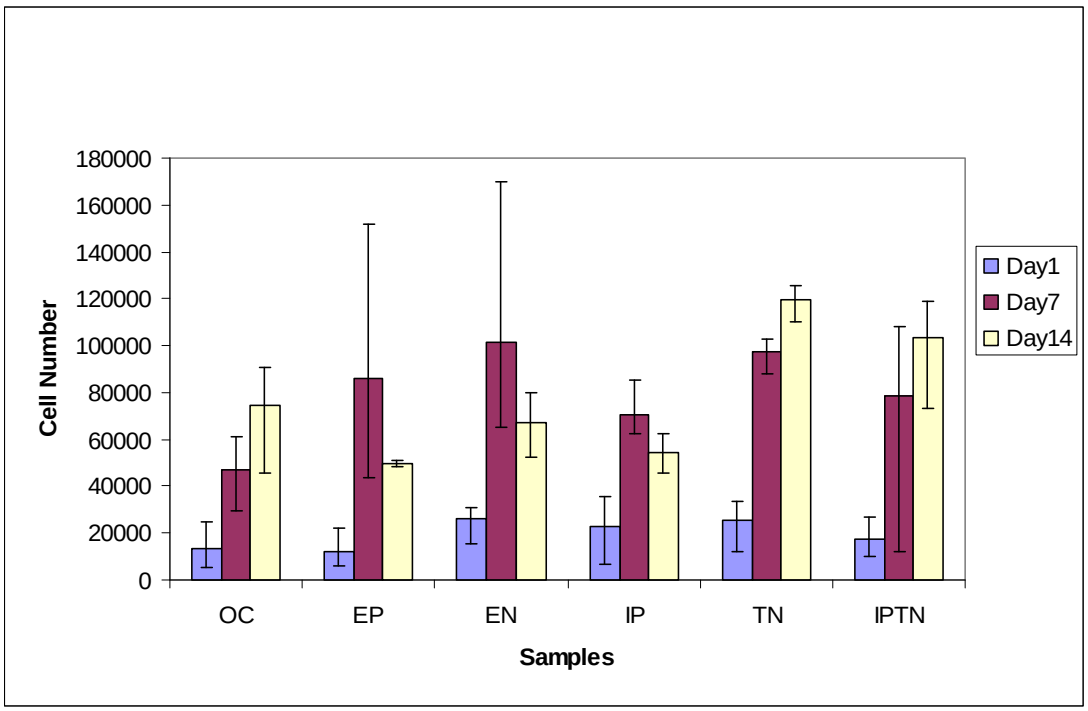


**Figure 3.3.** Cell growth determination in different culture medium throughout 14 days of incubation by MTS assay.

At the end of 1 day of incubation, all the cells with OC, EP, EN, IP, TN and IPTN showed approximately same cell numbers (around 10.000-20.000 cells/well). After 7 days in culture condition, there was a significant increase in the cell numbers in all the samples. Moreover, cell numbers in both EN and TN wells (around 100.000 and 95.000 cells/well, respectively) were observed to be higher than the others. At the end of 14 days of incubation, however, maximum number of cells was observed in cells with TN and IPTN (120.000 and 105.000 cells/well, respectively). Standard deviations of cells with EP and EN showed the highest scores.

### 3.2.4. Real Time-PCR

Collagen Type II expression of cells with different culture medium at the end of 1, 7 and 14 days of incubation was determined by relative Real Time PCR.

Real Time PCR analysis showed that Collagen Type II expression of cells in OC was increased throughout 14 days of incubation (Figure 3.4). Cells with IP showed similar expression pattern with OC. However, collagen expression of cells with EN and IPTN decreased after 14 days in culture.

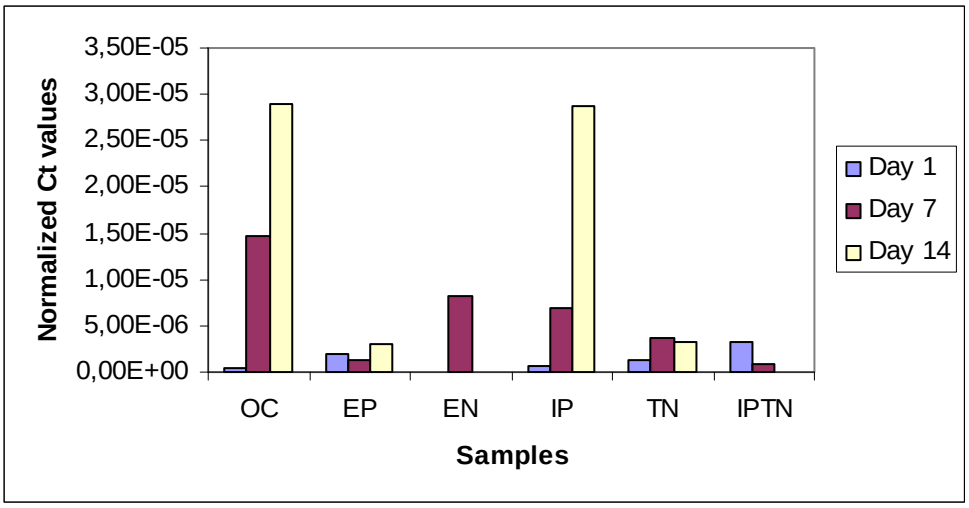


**Figure 3.4.** Collagen Type II expression of cells with different culture media at the end of 1, 7 and 14 days of incubation by relative Real Time PCR.

At the end of 1 day of incubation, all the cells with EP, EN, IP, TN and IPTN except OC showed minimal expression levels of Collagen Type II. After 7 days in culture condition, there was an increase in collagen expression in cells with OC, EN, IP, and TN. However, it was decreased in cells with EN and IPTN. On the other hand, highest expression was observed in cells with OC and IP at the end of 14 days of incubation.

### 3.2.5. Determination of ECM storage in Cell Culture Biochemical Methods - DMMB assay

sGAG formation of cells with different culture medium at the end of 1, 7 and 14 days of incubation was determined by DMMB assay.

DMMB assay showed that sGAG formation of cells in OC, EN, TN, and IPTN was increased throughout 14 days of incubation (Figure 3.5). However, it was decreased in cells with EP and IP after 14 days in culture.

At the end of 1 day of incubation, all of the samples showed minimal sGAG formation. After 7 days in culture, there was an increase in sGAG formation in cells with OC, EN, IP, and IPTN. However, it was decreased in cells with TN and the same in cells with EP. On the other hand, highest formation was observed in cells with TN at the end of 14 days of incubation. Formation of sGAG was higher in cells with TN and IPTN than OC.

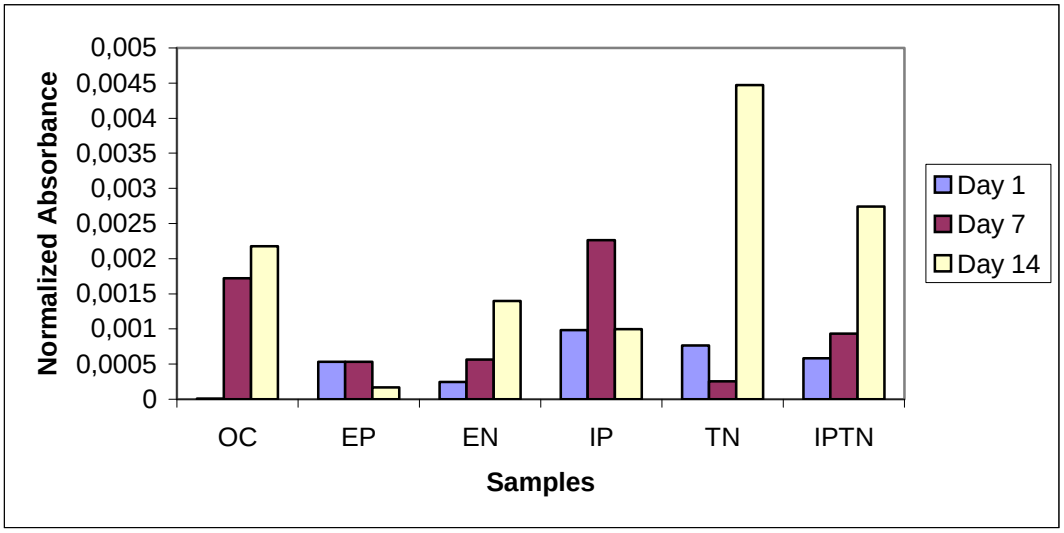


**Figure 3.5.** sGAG formation of cells with different culture medium at the end of 1, 7 and 14 days of incubation by DMMB assay.

### 3.2.6. Determination of ECM Storage in Cell Culture by Biochemical Methods-Hydroxyproline Assay

Collagen formation of cells with different culture medium at the end of 1, 7 and 14 days of incubation was determined by hydroxyproline assay.

Hydroxyproline assay demonstrated that collagen formation of cells in OC and IP was increased throughout 14 days of incubation (Figure 3.6). It was higher in cells with IP than with OC. However, it was decreased in the others in 14 days of culture.

At the end of 1 day of incubation, all of the samples showed minimal collagen formation but cells with IP was high. After 7 days in culture, there was an increase in collagen formation in all samples. Still, highest result was obtained from the cells with IP at day 7 as well as day 14.

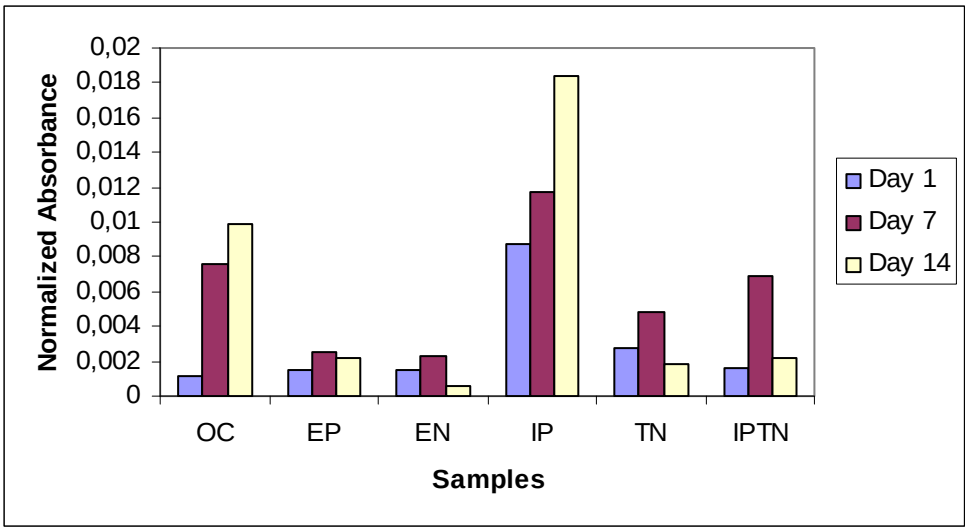


**Figure 3.6.** Collagen formation of cells in different culture media at the end of 1, 7 and 14 days of incubation as determined by hydroxyproline assay.

### 3.2.7. Determination of Cell Morphology in Cell Culture by Immunohistochemical Methods-Alexa Fluor® 546 Phalloidin and TO-PRO®-3 Staining for Confocal Microscopy

Cell morphology of cells incubated in different culture media at the end of 7 and 14 days of incubation was studied by confocal microscopy.

At the end of 7 days of incubation, especially cells in IPTN expressed chondrocyte morphology (Figure 3.7).

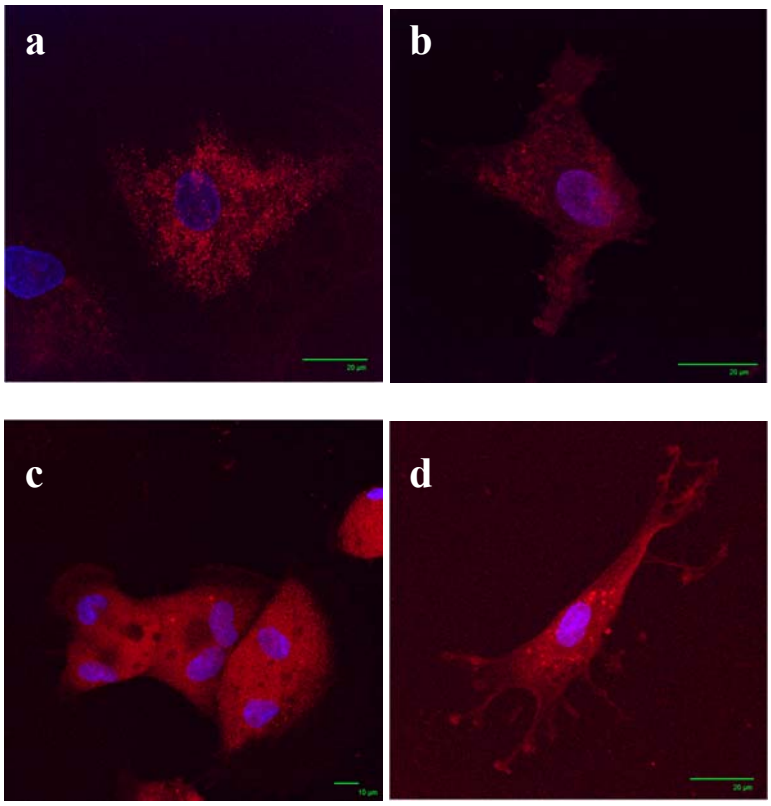


**Figure 3.7.** Cell morphology of cells in different culture media at the end of 7 days of incubation, obtained by confocal microscopy. (a) OC, (b) IP, (c) TN, and (d) IPTN. TO-PRO (blue), Phalloidin (red) (63X).

At the end of 14 days of incubation, cellular morphology did not change for the cells with OC, IP, TN and IPTN. (Figure 3.8).

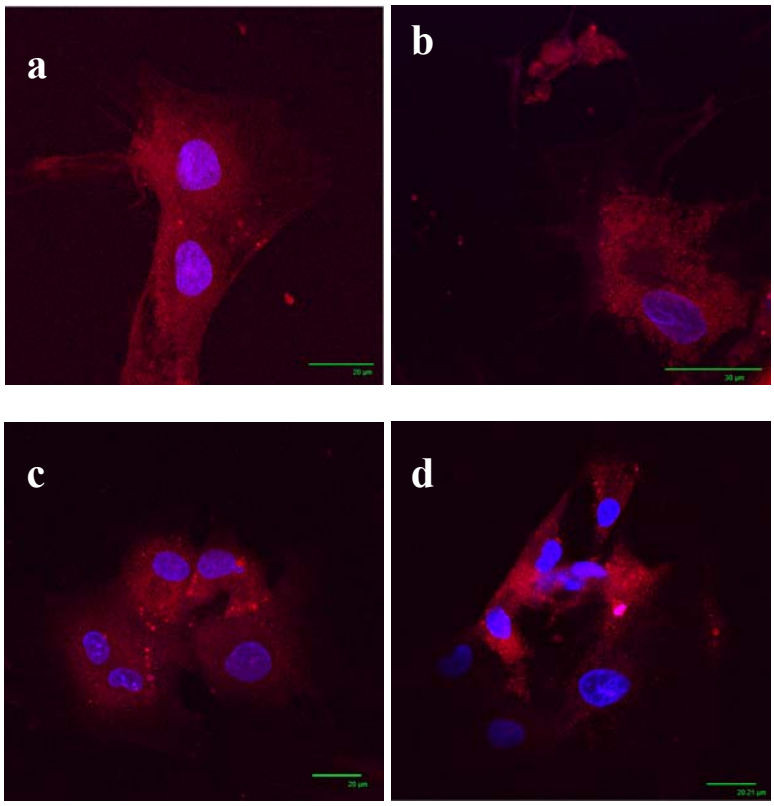


**Figure 3.8.** Cell morphology of cells in different culture media at the end of 14 days of incubation, as observed by confocal microscopy. (a) OC, (b) IP, (c) TN, and (d) IPTN. TO-PRO (blue), Phalloidin (red) (63X).

### 3.2.8. Determination of ECM Storage in Cell Culture by Immunohistochemical Methods-Collagen Type II and Aggrecan Confocal Microscopy Double Staining

ECM storage of cells with different culture medium at the end of 7 and 14 days of incubation was determined by collagen type II and aggrecan confocal microscopy double staining.

Articular cartilage was used as the positive control (Figure 3.9). At the end of 7 days of incubation, cells with OC demonstrated minimal ECM deposition. The cells with IP, TN and IPTN exhibited more ECM deposition (Figure 3.10).

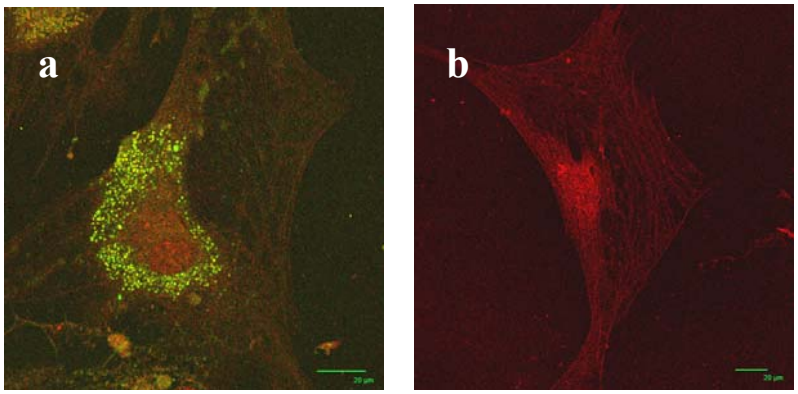


**Figure 3.9.** Collagen type II and aggrecan double staining of articular cartilage on Day 7 (a) collagen type II (green) and aggrecan (red) (b) aggrecan (red) (63X).

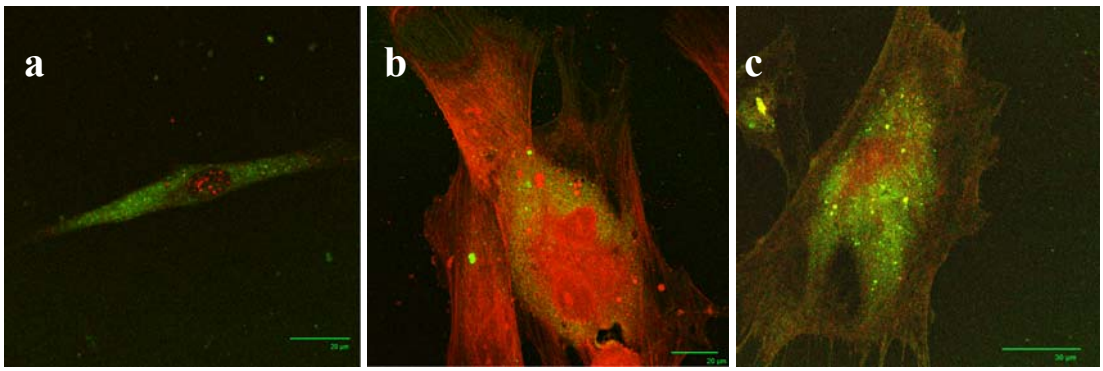


**Figure 3.10.** ECM storage of cells in different culture media at the end of 7 days of incubation by collagen type II and aggrecan double staining of (a) OC, (b) TN, and (c) IPTN. Collagen type II (green), aggrecan (red) (63X).

At the end of 14 days of incubation, ECM content of all cells with OC, IP, TN and IPTN was increased in comparison to that of day 7 (Figure 3.11). ECM storage was still higher in cells with IP, TN and IPTN than that of OC.

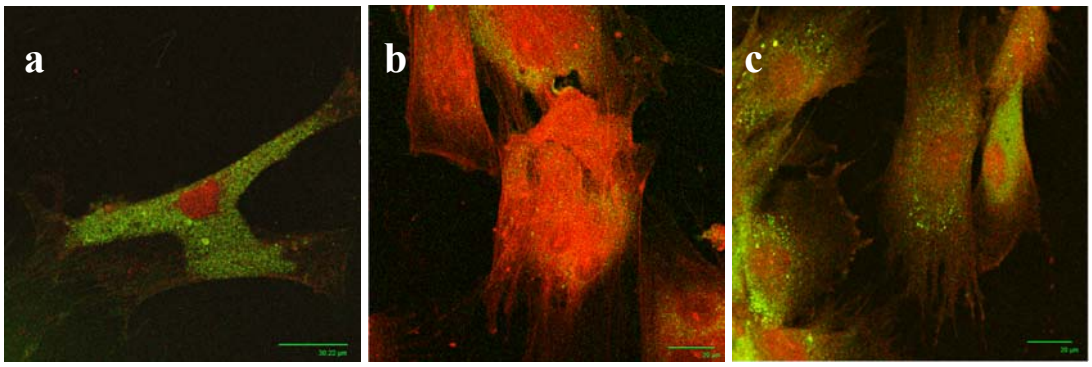


**Figure 3.11.** ECM storage of cells with different culture medium at the end of 14 days of incubation by collagen type II and aggrecan double staining of (a) OC, (b) TN, and (c) IPTN. Collagen type II (green), aggrecan (red) (63X).

### 3.3. *in situ* and *in vitro* Characterization of PLGA Sponge Scaffolds and Growth Factor Loaded Nanospheres with PLGA Sponge Scaffolds

#### 3.3.1. SEM of Empty Porous PLGA Scaffolds

The surface characteristics, average pore sizes and the pore distribution of the PLGA scaffolds were observed by SEM (Figure 3.12). By means of salt leaching method, the PLGA scaffolds were made porous. After salt leaching, the number of the pores inside the foams increased. In this study, salt crystals with a size range of 300-500 μm were used. It was observed from the micrograph that pores were homogenously distributed and their sizes were between 300-500 μm.

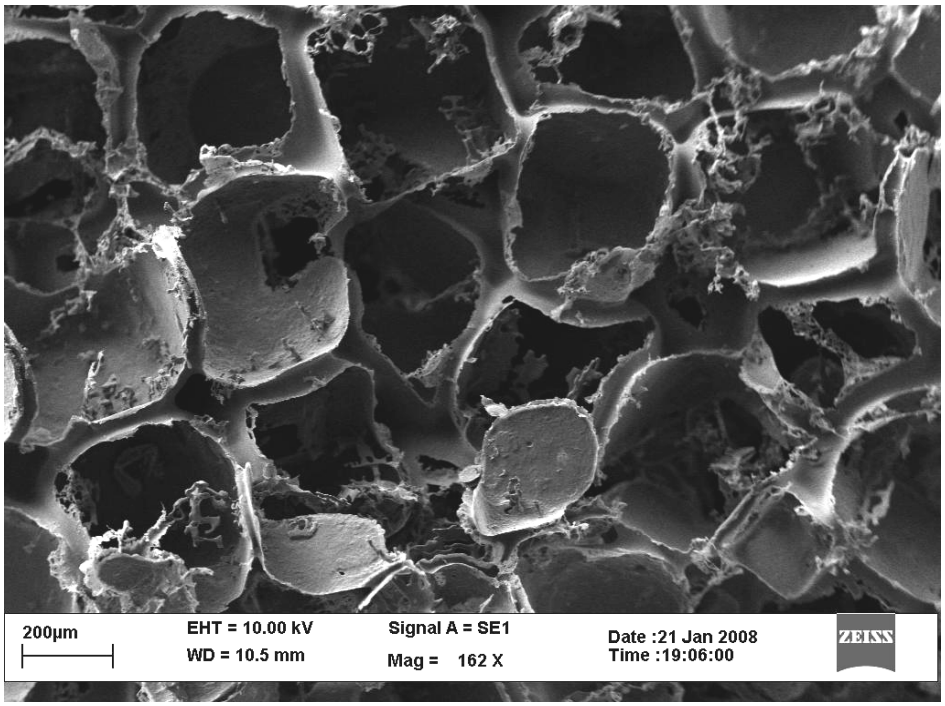


**Figure 3.12.** SEM of empty porous PLGA scaffolds prepared with 300-500 μm NaCl crystals.

#### 3.3.2. Porosity

Image analysis was carried out to characterize the constructs in terms of their porosity. More specifically the aim was to calculate the average pore size and the pore size distribution of the matrices. They were examined by using Quantachrome Poremaster for Windows Version 4.03.

Image analysis results showed that the porosity of the PLGA scaffolds was around 0.521. Their pore size distributions were homogeneous with the same diameter.

### 3.3.3. Degradation Study of Empty Porous PLGA Scaffolds

For the degradation study, empty porous PLGA foams were used. All measurements were expressed as means ± standard deviation (sd) relative to the initial values.

PLGA foams led to a pH decrease of 4.9 pH units in 120 days (Figure 3.13). At the end of 40 days of incubation more than 90% of the PLGA foams were degraded.

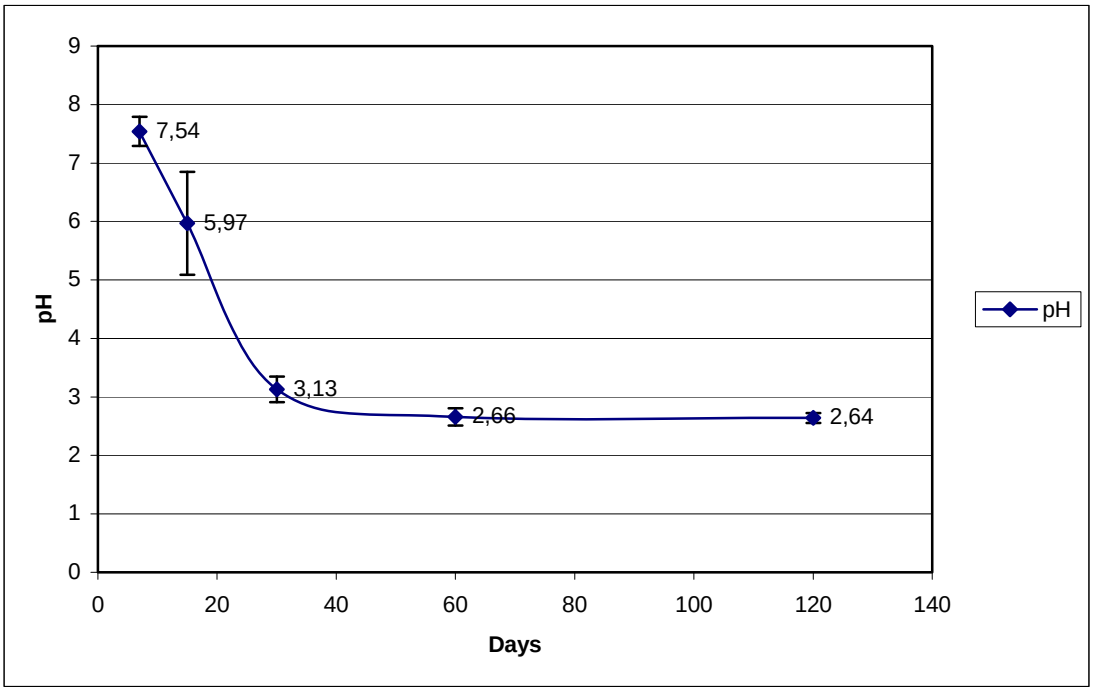


**Figure 3.13.** Degradation of PLGA scaffolds by change in pH.

### 3.3.4. Cell Growth on Cell Containing Porous PLGA Scaffolds

At the end of 1, 7 and 14 days of incubation in different culture media (Table 3.2) proliferation of bone marrow derived mesenchymal stem cells on PLGA scaffolds were measured by MTS assay.

**Table 3.2.** Abbreviations of the different culture media used on PLGA scaffolds.

| Abbreviation | Culture Medium |
|---|---|
| OC | only differentiation medium |
| CHA | chondroitin sulphate + alginate |
| EP | chondroitin sulphate + alginate + differentiation medium with empty PLGA nanospheres |
| EN | chondroitin sulphate + alginate + differentiation medium with empty NIPAM nanospheres |
| IP | chondroitin sulphate + alginate + differentiation medium with IGF-I loaded PLGA nanospheres |
| TN | chondroitin sulphate + alginate + differentiation medium with TGF- β1 loaded NIPAM nanospheres |
| IPTN | chondroitin sulphate + alginate + differentiation medium with IGF-I loaded PLGA nanospheres + TGF- β1 loaded NIPAM nanospheres |

The cell numbers in OC increased throughout 14 days of incubation (Figure 3.14). However, the amount of cells on CHA, EP, EN and IP (15.000, 25.000, 18.000 and 20.000 cells/well, respectively) first increased until the 7 days of incubation, then, abrupt decrease was observed in those samples at the end of 14 days in culture. Cell growth in TN and IPTN was decreased throughout 14 days of incubation.

At the end of 1 day of incubation, the cell number of all samples was lower from the initial cell seeding density which was 25.000 cells/well. Cells numbers of the samples EP and TN were higher (20.000 and 18.000 cells/well) than that of the other samples. After 7 days in culture, there was a significant increase in cell number in cells with OC, CHA, EP, EN, and IP (8.000, 15.000, 25.000, 18.000, and 20.000 cells/well, respectively). However, it was decreased in cells with TN and IPTN (15.000 and 8.000 cells/well). Standard deviations of cells with EP and EN showed the highest scores.

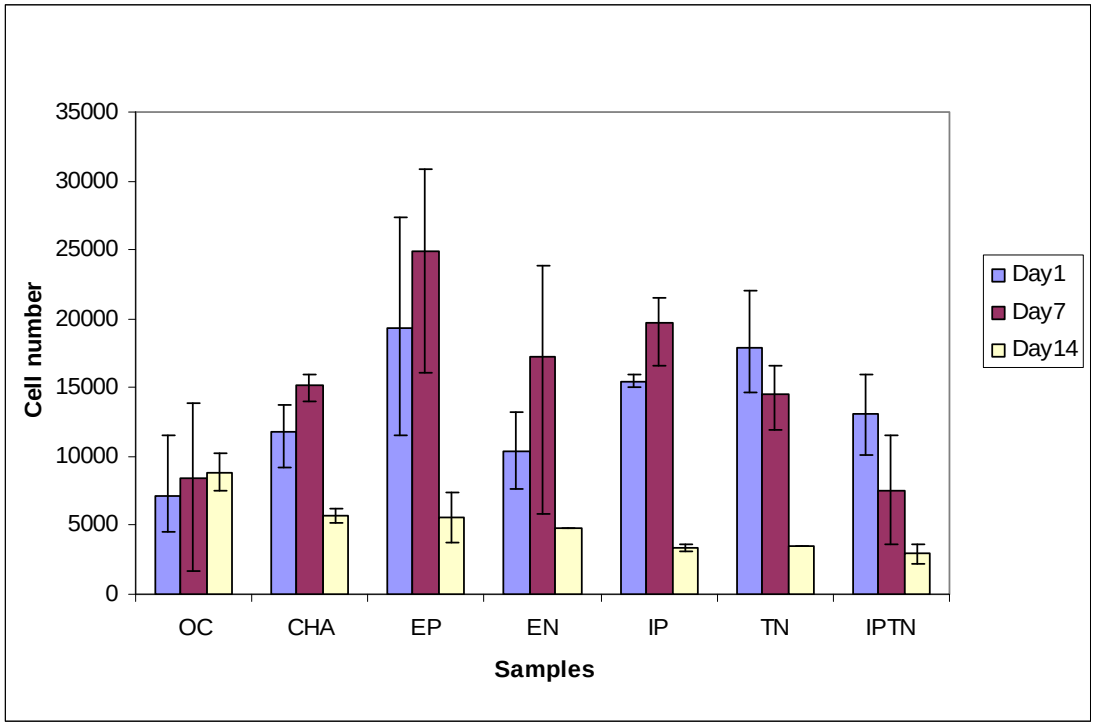


**Figure 3.14.** Cell growth determination in different culture medium throughout 14 days of incubation on PLGA scaffolds by MTS assay.

### 3.3.5. SEM of Cell Containing Porous PLGA Scaffolds

The surface characteristics and cell-surface interaction of cell seeded PLGA scaffolds were determined by SEM. The extent of degradation can be observed from the micrographs especially after 7 days of incubation

Scanning Electron Micrographs showed the presence and attachment of the cells on the walls of PLGA scaffolds throughout 14 days of incubation (Figure 3.15). They were found in close proximity of each other due to the initial application of the cells on small areas on the surface of the polymers especially at the end of 1 day of incubation.

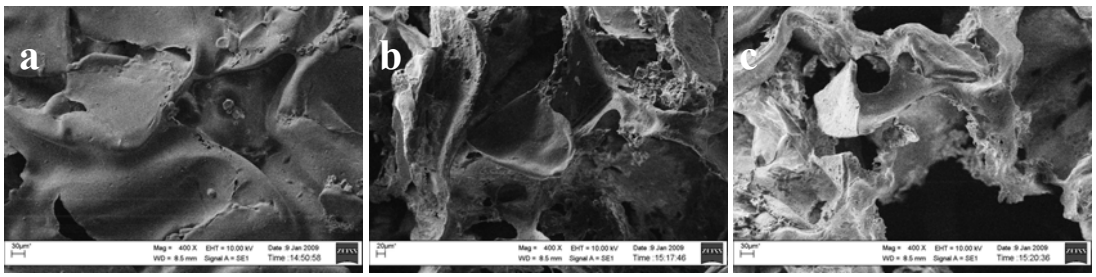


**Figure 3.15.** SEM of cells with CHA on PLGA scaffolds at the end of (a) 1 day, (b) 7 days, and (c) 14 days of incubation (400 X).

Figure 3.16 shows the cells grown in different culture media on PLGA scaffolds at the end of 7 days of incubation. The sizes of cells were approximately 10-30 μm in diameter as was expected.

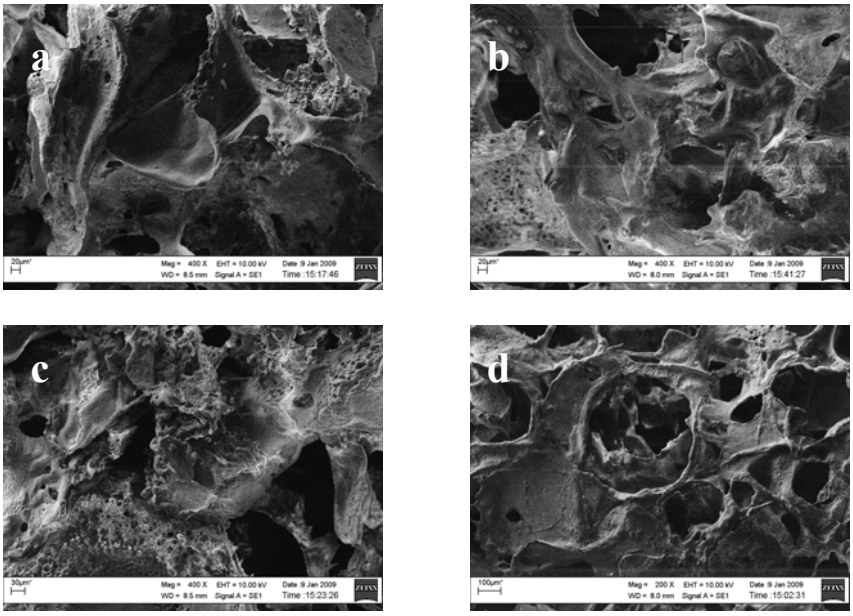


**Figure 3.16.** Scanning Electron Micrographs of cells with different culture medium on PLGA scaffolds at the end of 7 days of incubation (a) CHA (400X), (b) IP (400X), (c) TN (400X), (d) IPTN (200X).

### 3.3.6. Confocal Microscopy with Double Staining for Collagen Type II and Aggrecan Double Staining of Cell Containing Porous PLGA Scaffolds

Collagen production by cells grown in different culture media in PLGA scaffolds at the end of 7 and 14 days of incubation was shown by staining for collagen type II and aggrecan and examining under a confocal microscope.

Collagen production by cells grown in a medium containing IPTN increased gradually during the 14 days of incubation (Figure 3.17).

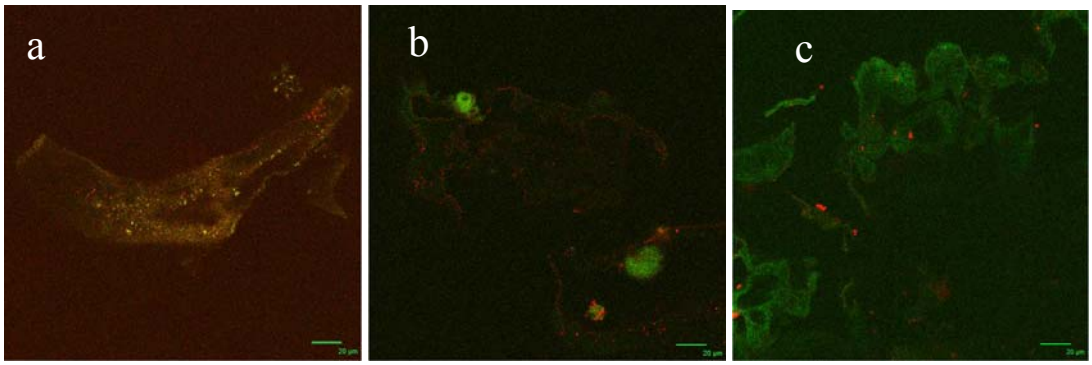


**Figure 3.17.** Collagen production by cells in PLGA scaffolds grown in IPTN medium at the end of (a) 1 day, (b) 7 days, and (c) 14 days of incubation. Staining was for collagen type II (green), aggrecan (red) (63X).

At the end of 14 days of incubation, collagen formation by cells in PLGA scaffolds grown in OC medium was found much more than that in TN and IPTN (Figure 3.18).

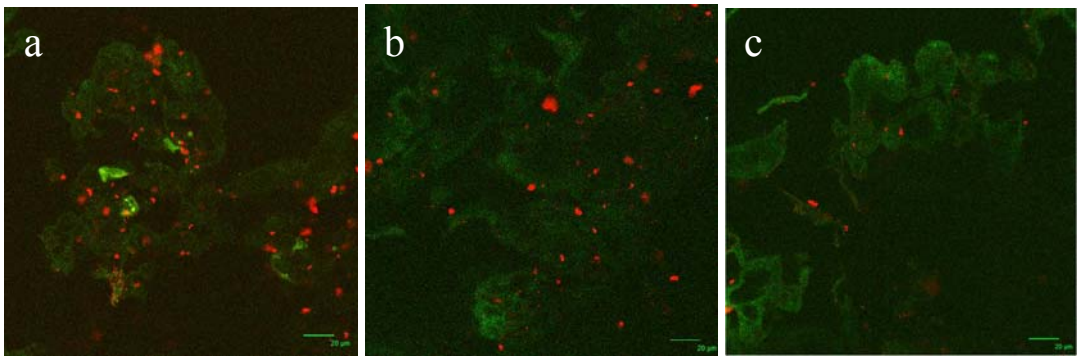


**Figure 3.18.** Collagen production by cells grown in different culture media in PLGA scaffolds at the end of 14 days of incubation. Staining was for collagen type II and aggrecan . Media (a) OC, (b) TN, and (c) IPTN. Collagen type II (green), aggrecan (red) (63X).

### 3.3.7. sGAG Production in Cell Loaded Containing Porous PLGA Scaffolds

sGAG production by cells grown in different culture medium into the PLGA scaffolds at the end of 1, 7 and 14 days of incubation was determined by alcian blue and nuclear fast red staining.

Alcian blue stains sGAG in the cells and if there are sGAG in the medium, the color turns to blue. Nuclear fast red staining stains the nucleus red. This test showed that at the end of 1 day of incubation, nuclei of all the samples were stained red. Then, after 7 days of incubation, blue staining was observed due to the presence of sGAG (Figure 3.19).


**Figure 3.19.** sGAG formation of cells with IP into the PLGA scaffolds as shown by alcian blue and nuclear fast red staining at the end of (a) 1 day, (b) 7 days, (c) 14 days of incubation (4X).

Figure 3.20 shows that at the end of 1 day of incubation, all of the samples were negative. After 7 days in culture, there was an increase in sGAG production in all the samples. Cells with IP and IPTN had the highest sGAG content. At the end of 14 days of incubation, the scores in all the samples had increased. Cells with IP gave the best result in this test. Level of sGAG formation in IPTN was found to be between IP and TN after 14 days of incubation.

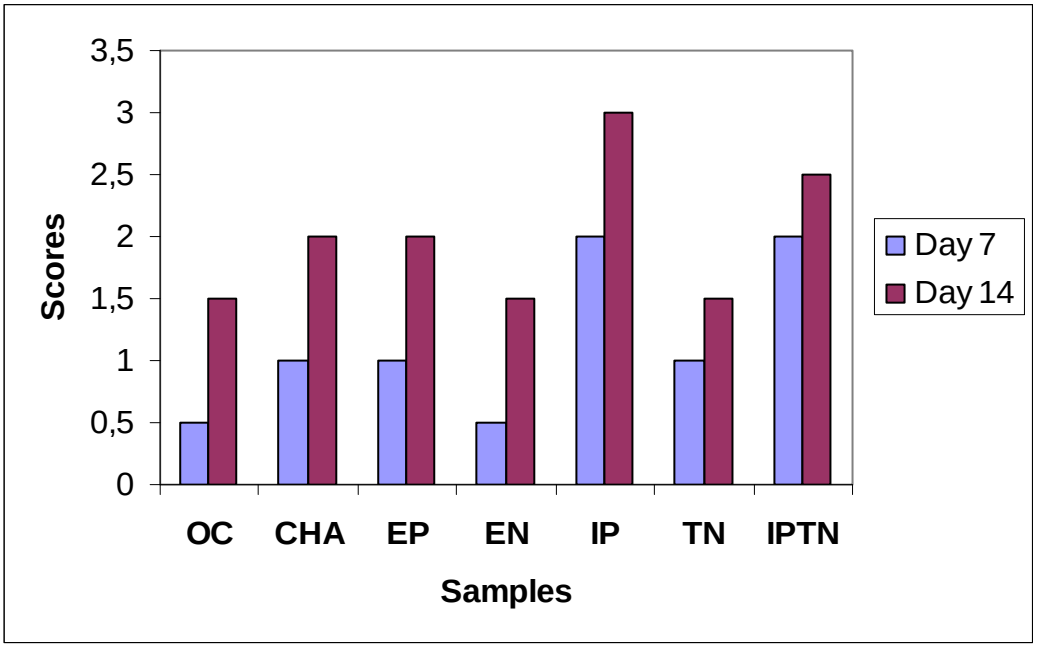


**Figure 3.20.** sGAG formation of cells with different culture medium into the PLGA scaffolds at the end of 7 and 14 days of incubation by alcian blue staining.

## 4. DISCUSSION

There are two major growth factors that forces cells to differentiate into chondrocytes, IGF-I and TGFβ-I. These growth factors were loaded in synthetic polymeric nanospheres that are pH sensitive PLGA and temperature sensitive NIPAM. In this study, mesenchymal cells (MSCs) were exposed to empty and growth factor loaded nanospheres both in the presence and absence of PLGA scaffold to provide differentiation of cells into chondrocytes.

There are two sets of experiments in this study, in the first part of the experiment (on TCPS), MTS tests were carried out to determine the density of cells grown in different media on TCPS. In order to find the exact cell number, a calibration curve was constructed. Cell numbers on all samples increased by time except those grown in EP and EN. The decrease in cell number of EP and EN might be due to the presence of the nanospheres. Addition of growth factors into the nanospheres reverted this pattern. TGF- β has an indirect mitogenic effect for certain MSCs and is a stimulator of extracellular matrix deposition (Kay et al., 1998, Moses et al, 1991, Lee et al, 2006). Since TGFβ-1 has a mitogenic activity on cells, increase in proliferation can be seen in both TN and IPTN. Since the cell growth rate of IPTN was found between IP and TN in 7 and 14 days of incubation, it could be the evidence for the cumulative effect of the growth factors released at the same time. Moreover, cell numbers in both EN and TN wells were observed to be higher than the others. Also, maximum growth rate was observed in cells with TN and IPTN at the end of 14 days of incubation. Samples incubated with NIPAM showed higher cellular growth than samples incubated in PLGA.

After observing the cell proliferation rate on TCPS, the differentiation of the cells was determined by real time PCR, DMMB and hydroxyproline assay and immunofluorescence methods. Collagen Type II is the major component of hyaline joint cartilage (Pulkkinen, 2008). Highest expression was observed due to differentiation in cells with OC and IP at the end of 14 days of incubation according to collagen type II expression results in real time PCR. Insulin-like growth factor I

(IGF-I) is known to stimulate collagen production (Olsen et al., 2007; Sonal, 2001). The reason why also cells with OC showed differentiation was that the medium of OC contains basal level of differentiation factors itself.

In order to support real time PCR, biochemical methods were also used for differentiation of cells. Collagen type II is produced and then secreted by chondrocytes, supporting chondrocyte adhesion and phenotypic differentiation of cells. It contains specifically proline and lysine amino acids and many modifications that are almost unique to collagen. These modifications such as hydroxylation of residues are introduced during biosynthesis eventually creating hydroxyproline. So the hydroxyproline assay gives information about the collagen production and cartilage differentiation. This assay shows that cells with IP produced highest collagen at the end of 7 and 14 days. Moreover, cells with IP showed higher score than OC for both time points. This data confirms the real time PCR data.

Another biochemical method was used to determine the ECM deposition is the measurement of sGAG formation. The test showed that sGAG formation of cells in OC, EN, TN, and IPTN was increased during the 14 days of incubation. ECM storage is important in cartilage formation. The highest levels of sGAG formation were observed in cells with TN and IPTN. There are several reports demonstrating that TGF-β1 supports the aggrecan deposition by regulation of aggrecanase inhibitor. (Kudo et al., 2001; Yamanishi et al., 2002). Poleni et al. (2007) found that TGF-β1 increased GAGs' content and deposition in rat cartilage cells by 3.5-fold and six-fold, respectively, and induced aggrecan expression around 10-fold. The amount sGAG production in IPTN containing medium was found to be between IP and TN in 7 and 14 days of incubation, showing the combined effect of the two growth factors simultaneously.

Cell morphology, Collagen Type II and aggregan deposition were measured by confocal microscopy. First, cells were stained with Alexa Fluor® 546 Phalloidin for actin filaments, and TO-PRO®-3 for nuclei to have information about cell morphology. TGFβ-1 containing cells were observed to be highly proliferative. This result supports the MTS assay results.

ECM storage of cells with different culture medium at the end of 7 and 14 days of incubation was determined in confocal microscopy by collagen type II and aggrecan

double staining. In this study, articular cartilage cells were used as a positive control. It was observed that collagen type II was deposited around the nucleus. However, cells with OC did not express the same behavior. Collagen type II was distributed all over the cytoplasm. On the other hand, cells with IP, TN and IPTN showed similar accumulation patterns of collagen type II with articular cartilage cells. Moreover, cells with TN showed high amount of aggrecan deposition which supports the data of DMMB assay.

In the second part of the study (on scaffold), first the porosity and degradation rate of PLGA scaffold were studied. The pore size and the pore distribution are quite important parameters for cell growth inside the matrices. Since cells cannot grow without cell to cell contact, the average size of the pores should be at least 3 times bigger than that of the cells so that a single cell can find a chance to establish contact with the others. SEM micrographs showed that pores were homogenously distributed and their sizes were between 200-400 μm, that are large enough for cell penetration and growth. Degradation study showed that at the end of 40 days of incubation, more than 90% of the PLGA foams were degraded. Degradation of PLGA is followed by pH decreases due to lactic acid release and therefore, the sample that leads to the largest pH drop would have been the one that has degraded the most.

Cell growth on porous PLGA scaffolds was determined by MTS assay. During initial seeding, cells did not attach onto the PLGA scaffold surface properly. They might prefer to grow onto TCPS. The maximum cellular attachment was observed in cells with EP. The number of the cells with TN and IPTN decreased at day 7: Cells might prefer to undergo differentiation more than proliferation due to the effect of growth factors. Cells can not grow and differentiate at the same time. One reason of the decrease in the cell number of all samples except OC at the day of 14 could be due to the rapid degradation of the PLGA. The other reason could be the absence of chondroitin sulphate in cells with OC. Chondroitin sulphate is an essential component of cartilage tissue, so it could lead to cellular differentiation. Because of that reason, cell number of the other samples except OC might droped at the end of 14 days of incubation.

SEM was used to determine the surface characteristics and cell-surface interaction of the cell seeded PLGA scaffolds. From these micrographs, one can not decide which culture medium is ideal for the MSC differentiation into cartilage since only a small

fraction of the polymers were examined under the SEM. In these aspects immunochemical data are expected to be more conclusive.

Collagen formation of cells in different culture medium on PLGA scaffolds at the end of 7 and 14 days of incubation was determined by collagen type II and aggrecan double staining and confocal microscopy examination. Collagen formation of cells with IPTN into the PLGA scaffolds was increased throughout 14 days of incubation.

Cells seeded in PLGA scaffolds in the presence of different culture medium were stained with alcian blue to determine sGAG formation at 1, 7 and 14 days of incubation. Alcian blue stains both sulfated and carboxylated acid mucopolysaccharides and sulfated and carboxylated glycoproteins. The rate of alcian blue staining score is directly correlated with cellular differentiation into the cartilage. At the end of 1 day of incubation, the cell nucleuses were observed as red color by nuclear fast red stain. There was no blue color seen due to the fact that cells did not expressed sGAG component. At day 7, samples stained with alcian blue as was expected. The highest scores were observed by the cells with IP and IPTN at both day 7 and 14. The samples with CHA showed higher sGAG formation than OC on day 7 and 14. Moreover, there was no effect of cells with EP on the cellular sGAG formation. In the case of cells with EN, sGAG formation was lower than that of CHA. The reason could be the suppressive effect of EN on the expression of sGAG. sGAG formation rate in IPTN was again found between IP and TN in 14 days of incubation.

## 5. CONCLUSION

Mimicking the structure and function of the natural ECM is one of the most important requirements in tissue engineering. At the end of the study, the interaction between cells and biodegradable nanospheres has been identified. The assays showed that, the release of growth factors affect both the cells on TCPS and in the scaffolds. Responsive nanospheres were used in the controlled release of GFs for chondrocyte-like cell development in scaffolds. Biochemical and immunofluorescence analysis gave information on the success of cell differentiation. At the end of the study, it can be concluded that cells with IP and IPTN nanospheres give the best results with respect to cartilage differentiation onto scaffolds. It thus appears that, usage of growth factor loaded nanospheres could have a serious contribution on cartilage tissue engineering. In future studies, the physiological aspect of this interaction could also be investigated to find out the effects of these nanospheres on cells in vivo. Also, the responsiveness of these nanospheres needs to be tested to control the release of the growth factors within the scaffolds.

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**CURRICULUM VITAE**

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|---|---|
| **Candidate's full name:** | Ayşe Burcu Ertan |
| **Place and date of birth:** | İstanbul - 17.08.1982 |
| **Permanent Address:** | Bahçelerarası sk. Tan apt.<br>23/7 Boıstancı/İstanbul |
| **Universities and Colleges attended:** | Haliç University - Molecular Biology and Genetics<br>İstanbul Teknik University - Molecular Biology-Genetics and Biotechnology |