Nuseybe AĞIRMAN

INVESTIGATION OF CHONDROGENIC PROPERTIES OF BONE MARROW DERIVED MESENCHYMAL STEM CELLS ON PAMAM DENDRIMER INCORPORATED CHITOSAN SCAFFOLDS

FATİH UNIVERSITY

The Graduate School of Sciences and Engineering

Master of Science in
Biology

INVESTIGATION OF CHONDROGENIC PROPERTIES OF BONE MARROW DERIVED MESENCHYMAL STEM CELLS ON PAMAM DENDRIMER INCORPORATED CHITOSAN SCAFFOLDS

by

Nuseybe AĞIRMAN

May 2013

M.S.
2013

INVESTIGATION OF CHONDROGENIC PROPERTIES OF BONE MARROW DERIVED MESENCHYMAL STEM CELLS ON PAMAM DENDRIMER INCORPORATED CHITOSAN SCAFFOLDS

by

Nuseybe AĞIRMAN

A thesis submitted to

The Graduate School of Sciences and Engineering

of

Fatih University

in partial fulfillment of the requirements for the degree of

Master of Science

in

Biology

May 2013
Istanbul, Turkey

APPROVAL PAGE

This is to certify that I have read this thesis written by Nuseybe AĞIRMAN and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in Biology.

_____
Assoc. Prof. Dr. Sevim IŞIK
Thesis Supervisor

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science in Biology.

_____
Assoc. Prof. Dr. Lokman ALPSOY
Head of Department

Examining Committee Members

Assoc. Prof. Dr. Sevim IŞIK _____

Assoc. Prof. Dr. Lokman ALPSOY _____

Asst. Prof. Dr. Mehmet ŞENEL _____

It is approved that this thesis has been written in compliance with the formatting rules laid down by the Graduate School of Sciences and Engineering.

_____
Assoc. Prof. Dr. Nurullah ARSLAN
Director

May 2013

INVESTIGATION OF CHONDROGENIC PROPERTIES OF BONE MARROW DERIVED MESENCHYMAL STEM CELLS ON PAMAM DENDRIMER INCORPORATED CHITOSAN SCAFFOLDS

Nuseybe AĞIRMAN

M.S. Thesis – Biology
May 2013

Thesis Supervisor: Assoc. Prof. Dr. Sevim IŞIK

ABSTRACT

Tissue engineering and regenerative medicine is the new light for the damaged tissues and lost organs that cannot be treated with other medical methods. With the developing technologies in the stem cell biology and the natural biocompatible materials, functional artificial tissues and organs can be produced.

Mesenchymal stem cells (MSCs) with their ability to differentiate into different lineages of cells can be used in tissue regeneration. They make a good candidate for tissue engineering studies because of their immunosuppressive properties and ease of isolation, and they are ethical to use in research.

Appropriate scaffold is one of the basic components in tissue engineering studies. The scaffold can be produced either from natural or synthetic materials. Some of the most common synthetic materials are polyactic acid (PLA), polyglycolic acid (PGA) and poly-lactic-co-glycolic acid (PLGA) whereas some of the most common natural materials are chitosan, silk and collagen. Chitosan is a natural polysaccharide that mimics the glycosaminoglycans (GAGs) present in native cartilage extracellular matrix (ECM) and exhibits chondrogenic properties. CTS is versatile, biodegradable and non-toxic material that allows it to be one of the best candidate for scaffold production for chondrogenic tissue engineering.

In this study, five different CTS based scaffolds including scaffolds with incorporation of PAMAM dendrimers – namely CTS (only chitosan), G0, G1, G2 and G3 (1,2,3 and 4 generations of PAMAM incorporated, respectively) were synthesized

for studying chondrogenic tissue. Scanning electron microscope (SEM) imaging of scaffolds were done and characterization tests applied. Biodegradability of the scaffolds was tested with lysozyme treatment. Cytotoxicity and proliferation experiments were analyzed by LDH assay and WST-1 Assay respectively. Human bone marrow MSC line was used for ease of availability and chondrogenic differentiation of these cells was conducted in monolayer culture successfully.

As the result of this study, PAMAM incorporated chitosan scaffolds were more biodegradable than only chitosan scaffold with four weeks of lysozyme treatment. The only chitosan scaffold showed 10% weight loss whereas most branched G3 scaffold showed about 20% weight loss. Also all types of scaffolds were found to be biocompatible in WST-1 and LDH assays. By taking only chitosan scaffold results as reference point, G3 scaffold showed the most proliferative result when compared to only chitosan scaffold. And in LDH assay, G3 scaffold was found to have very low toxicity even less than the low level toxicity of only chitosan scaffold.

The results showed that PAMAM incorporated chitosan scaffold have potential for studying cartilage tissue engineering. The most PAMAM incorporated G3 scaffold showed the best results. For future studies, we are planning to study and understand cell behavior on scaffolds in detail with molecular level studies and carrying on with the *in vivo* trials aiming for pre-clinical studies.

Keywords: Human Bone Marrow Mesenchymal Stem Cell Line, Chitosan, Tissue Engineering, Chondrogenic Differentiation, Scaffold Design, PAMAM dendrimer, PAMAM incorporation, Cartilage Tissue Engineering.

İNSAN KEMİK İLİĞİ KAYNAKLI MEZENKİMAL KÖK HÜCRELERİNİN PAMAM DENDRİMER EKLENMİŞ KİTOSAN DOKU İSKELELERİ ÜZERİNDEKİ KONDROJENİK ÖZELLİKLERİNİN İNCELENMESİ

Nuseybe AĞIRMAN

Yüksek Lisans Tezi – Biyoloji
Mayıs 2013

Tez Danışmanı: Doç. Dr. Sevim IŞIK

ÖZ

Doku mühendisliği ve rejeneratif tıp, tedavi edilemeyen dokular ve organ kayıpları için bilim dünyasında yeni bir kapı açmıştır. Kök hücre ve materyal biliminin gelişen teknolojileri ile fonksiyonel yapay dokular üretilebilmektedir.

Mezenkimal kök hücreler (MKH) birden çok hücre tipine dönüşebilmeleri sayesinde doku mühendisliğinde kullanılabilmektedirler. İmmunosupresif özellikleri ile izolasyon ve teminindeki kolaylık dolayısıyla onları doku mühendisliği için iyi bir adaydırlar ve bilimsel çalışmalarda kullanılmaları etik açıdan uygundur.

Uygun bir doku iskelesi doku mühendisliği çalışmalarının temel parçalarından biridir. Doku iskelesi, doğal ya da sentetik materyallerden üretilebilir. Sık kullanılan sentetik materyallerden bazıları poli(laktik-ko-glikolik asit) (PLGA), polilaktik asit (PLA) ve poliglikolik asit (PGA) iken sık kullanılan doğal materyallerden bazıları kitosan, ipek ve kolajendir. Kitosan, kıkırdak dokunun ekstraselüler matriksinde bulunan glikozaminoglikanları (GAG) mimik eden ve kondrojenik özellikler gösterebilen doğal bir polisakkarittir. Kitosan, kullanışlı, biyobozunabilir ve toksik olmayan bir madde olup kıkırdak doku mühendisliği amaçlı doku iskelesi üretimi için uygun bir seçenektir.

Bu çalışmada, kıkırdak doku mühendisliğinde kullanılma amaçlı doku iskeleleri oluşturulmuş olup, PAMAM dendrimer eklenmiş dört çeşit de içinde olmak üzere, isimleri CTS (sadece kitosan), G0, G1, G2 ve G3 (sırasıyla 1,2,3 ve 4 jenerasyon PAMAM dendrimer eklenmiş) şeklindedir. Bu doku iskelelerinin taramalı elektron

mikroskobu ile görüntüleri alınmış ve karakterizasyon testleri yapılmıştır. Lizozim enzimi uygulanarak biyobozunurlukları test edilmiştir. Proliferasyon ve sitotoksisite deneyleri sırasıyla LDH ve WST-1 testleri ile analiz edilmiştir. İnsan kemik iliği MKH hattı kültür şartları açısından uygun olması sebebiyle edilmiş kullanıldı ve hücre kültüründe kondrojenik hücreye dönüşümü başarıyla sağlandı.

Bu çalışmanın sonucunda, dört haftalık lizozim uygulaması sonrasında PAMAM eklenmiş kitosan doku iskeleleri sadece kitosan olarak üretilen doku iskelesinden daha biyobozunur olarak gözlendi. Sadece kitosan doku iskelesi %10 ağırlık kaybı gösterirken, en çok PAMAM dendirmer jenerasyonu eklenmiş G3 kitosan doku iskelesi %20 ağırlık kaybı gösterdi. Tüm doku iskelesi tipleri WST-1 ve LDH testlerinde biyouyumlu olarak gözlendi. Sadece kitosan doku iskelesi, CTS, referans noktası olarak alınarak diğer doku iskeleleri ile kıyaslandığında G3 kitosan doku iskelesi en çok proliferasyon gösteren doku iskelesi oldu. LDH sitotoksisite testinde ise, G3 kitosan doku iskelesinin, az toksisite gösteren CTS ile karşılaştırıldığında daha da düşük toksisiteye sahip olduğu gözlendi.

Sonuçlar, PAMAM eklenmiş kitosan doku iskelelerinin kıkırdak doku mühendisliği çalışmak için potansiyeli olduğunu göstermiştir. En fazla PAMAM dendrimer jenerayonu eklenmiş olan G3 kitosan doku iskelesi en iyi sonuçları vermiştir. Gelecek çalışmalarda, moleküler düzeydeki deneylerle hücrelerin doku iskelesi üzerindeki davranışını detaylıca çalışmayı planlıyoruz ve klinik öncesi çalışmalar için bu doku iskelelerinin *in vivo* denemelerini yapmayı planlıyoruz.

Anahtar kelimeler: İnsan Kemik İliği Mezenkimal Kök Hücre Hattı, Kitosan, Doku Mühendisliği, Kondrojenik Farklılaşma, Doku İskelesi Dizaynı, PAMAM Dendrimer, PAMAM Modifikasyonu, Kıkırdak Doku Mühendisliği.

*To my precious parent*s…

ACKNOWLEDGEMENT

I would like to express my appreciation to my supervisor Assoc. Prof. Dr. Sevim IŞIK and Asst. Prof. Dr. Mehmet ŞENEL from Chemistry Department for guiding me on experimental assays and giving me a new insight on research. I also would like to thank to all of my instructors in Fatih University.

I would like to thank my lab-mates Hakan ATAK, Rukiye KARABACAK, Tuğba SAĞIR, Şule TERZİOĞLU, Merve ZAİM and Aysel KARAGÖZ. I would specially thank Berna BÜYÜK from Chemistry Department for synthesis of scaffolds. Also to all my school and non-school friends for their support and friendship during and after our master education.

I acknowledge and express my gratitude to very supportive and generous TÜBİTAK that supported me with graduate scholarship for my master study years. Having a responsibility for graduate education was also a motivation to finish my studies.

Above all, I express my deepest gratitude to my precious family, my mother Azime AĞIRMAN and my father Osman AĞIRMAN. They were always with me with their endless love, support and patience. They made everything easier when things get hard in this journey. I could not be able finish this research without the support of them. Thank you.

TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURE

LIST OF SYMBOLS AND ABBREVIATIONS

ABBREVIATION

ASC	Adult stem cells
BM-MSC	Bone marrow derived mesenchymal stem cell
CTS	Only chitosan scaffold
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
EA	Water absorption
ECM	Extracellular matrix
EDA	Ethylenediamine
ES	Embryonic stem (cell)
ESC	Embryonic stem cell
FACS	Flourosence assisted cell sorting
FBS	Fetal bovine serum
G0	1st generation PAMAM incorporated chitosan scaffold
G1	2nd generation PAMAM incorporated chitosan scaffold
G2	3rd generation PAMAM incorporated chitosan scaffold
G3	4th generation PAMAM incorporated chitosan scaffold
GAG	Glicosaminoglycan
hMSC	Human mesenchymal stem cell
HSC	Hematopoietic stem cells
iPS	Induced pluripotent stem (cells)
LDH	Lactate dehydrogenase
MSC	Mesenchymal stem cell
PAMAM	Poly(amidoamine)
PBS	Phosphate base saline
PGA	Poly-glycolic acid

PLA	Poly-lactic acid
PLGA	Poly-lactic-co-glycolic acid
RNA	Ribonucleic acid
SEM	Scanning electron microscope

CHAPTER 1

INTRODUCTION

1.1 REGENERATIVE MEDICINE AND TISSUE ENGINEERING

1.1.1 Background Overview

In 1900s scientists take interest into maintenance of body organs *ex vivo*. In 1930s, Alexis Carrel and Charles Lindbergh studied *ex vivo* culture of organs. Because whole human organ failures were great importance in medicine, organ maintenance and transplantation were always desired to achieve. In 1955, first organ transplantation was achieved with kidney between identical twins. In 1960s, kidney transplantation by Murray was achieved between two genetically unrelated human [1-4]. Although Murray's study also showed that immune response could be overcome but it is still challenging today. Rarity of suitable donor, lack of donor number compared to organ-failed patients, possible immune reaction between donor and recipient and difficulty of controlling rejection of the tissue in recipient are still posing problems for transplantation in medicine. So scientists directed their ways to alternative treatments for such cases. Synthetic materials and designs that can substitute such failing organs and structures in the body are studied and developed in time with rising technology; medical prosthesis is an example for such designs. But these alternative ways were still not in full efficiency in substituting for the functional organs. Over time, with increased research areas *in vitro*, cell biology and cell culture techniques have also been developed [2]. Cell transplantation of bone marrow was achieved in 1970s [1], and human ESCs were isolated in 1998 [1,5]. The concept of transplantation, which aims maintaining native tissue functions in essence, by covering these new research areas - including stem cells, material science, cell transplantation and culture techniques and

regeneration and engineering defined as “regenerative medicine” by William Haseltine in in 1999 [1,6].

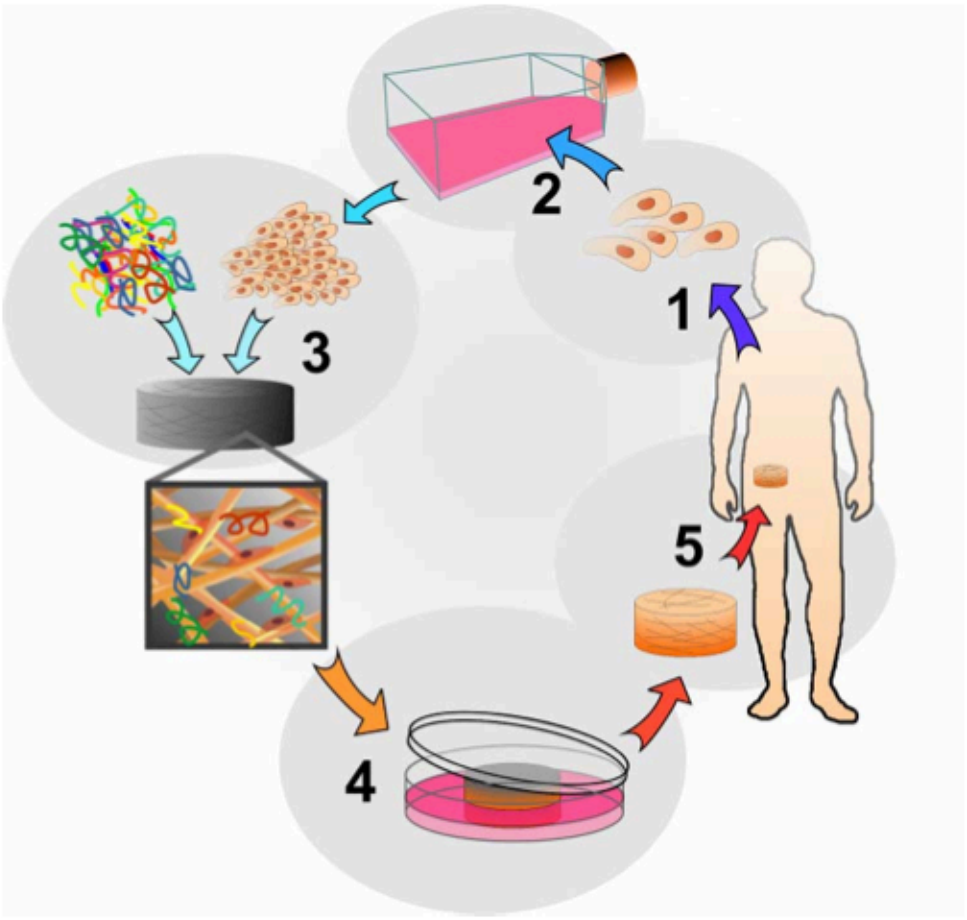


Figure 1.1 A scheme for basic tissue engineering protocol. 1) A sample of cells is isolated from a suitable source from the body. 2) The cells are cultured *in vitro* for proliferation and expansion. 3) Expanded cells are seeded onto designed scaffolds with biofactors that would create the suitable microenvironment. 4) Further expansion of cells and possible formation of tissue-like structure on designed scaffolds in culture is achieved. 5) Designed tissue like structure is generated *in vitro* and is ready for clinical transplantation [7].

1.1.2 Tissue Engineering

Tissue engineering, which the concept was first reported by Langer and Vacanti in 1993, is a field that combines the potential of material science and cell biology and biochemical substances for the treatment and regeneration of damaged tissues and organs, as if designing an artificial system that can substitute body parts or systems [8]. Tissue engineering has three main components for to study; cells, scaffolds and an optimal microenvironment mainly provided by biofactors (Figure 1.1) [8-9]. Appropriate cells should be obtained from a suitable source and should prone to grow *in vitro* culture. Scaffolds should provide the mechanical and structural support while being biocompatible, biodegradable, non-toxic and attachable by the desired cells [9]. After cells are isolated and proliferated *in vitro*, they are seeded onto scaffolds to further grow and proliferate in order to form a three-dimensional structure that could mimic the original tissue in the body with the provided microenvironment, which will mimic the original tissue, for the regeneration of desired tissue type [9]. If the three-dimensional structure is achieved and is functional then this *in vitro* generated tissue could then be transplanted for therapy of damaged or lost tissues or organs.

Figure 1.2 **“**Vacanti Mouse”, cartilage formation on scaffold implemented in the mouse [10].

Starting with a engineered cartilage in shape of human ear in 1992 by Vacanti et al. (Figure 1.2), tissue engineering has progressed in time with studies in various tissue types such as bladder (Figure 1.3) [1], heart (Figure 1.4) [11-12], trachea (Figure 1.5) [13] and skin (Figure 1.6) [14], yet it also keeps trying to overcome challenges in this area with each study.

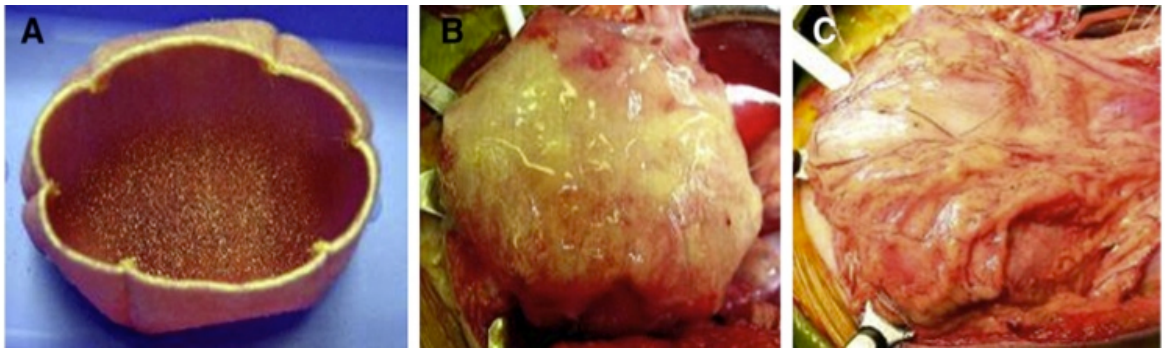


Figure 1.3 Tissue engineered bladder on collagen scaffolds [1].

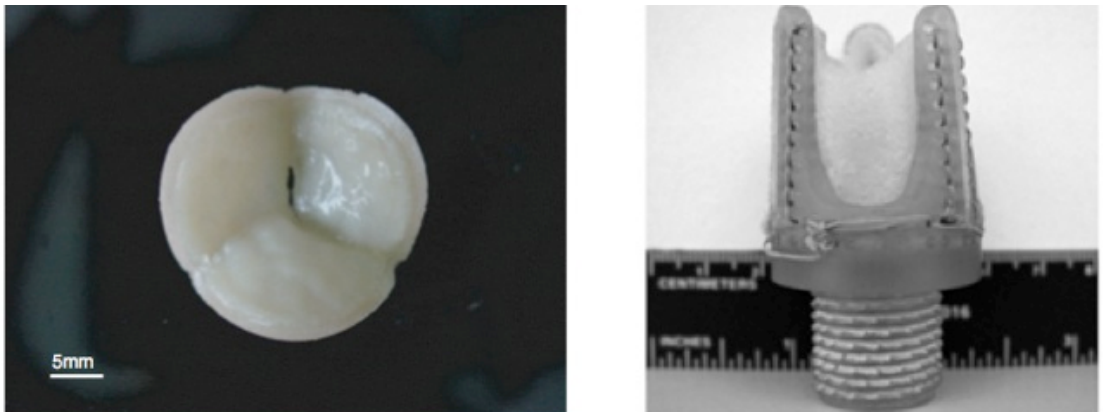


Figure 1.4 Tissue engineered heart valve (*on the left*) [11], and tissue engineered pulmonary valve (*on the right*) [12].

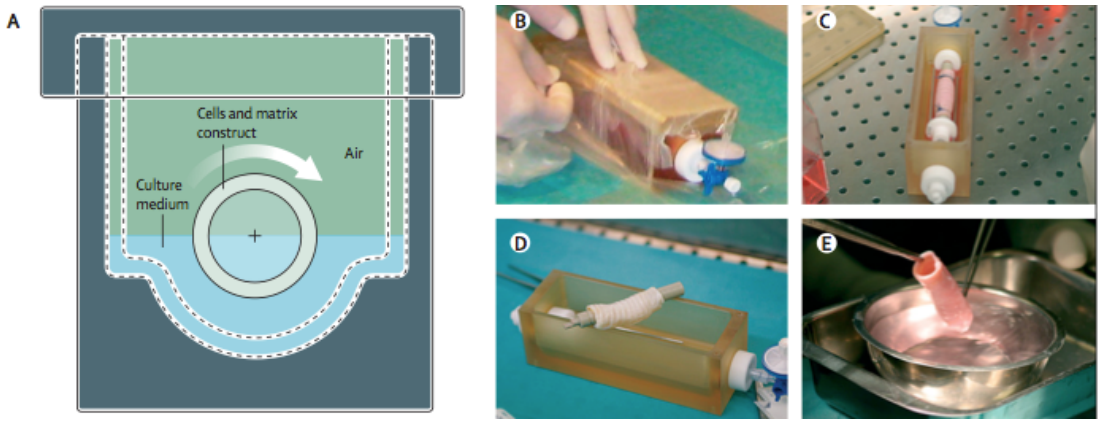


Figure 1.5 Tissue engineered trachea [13].

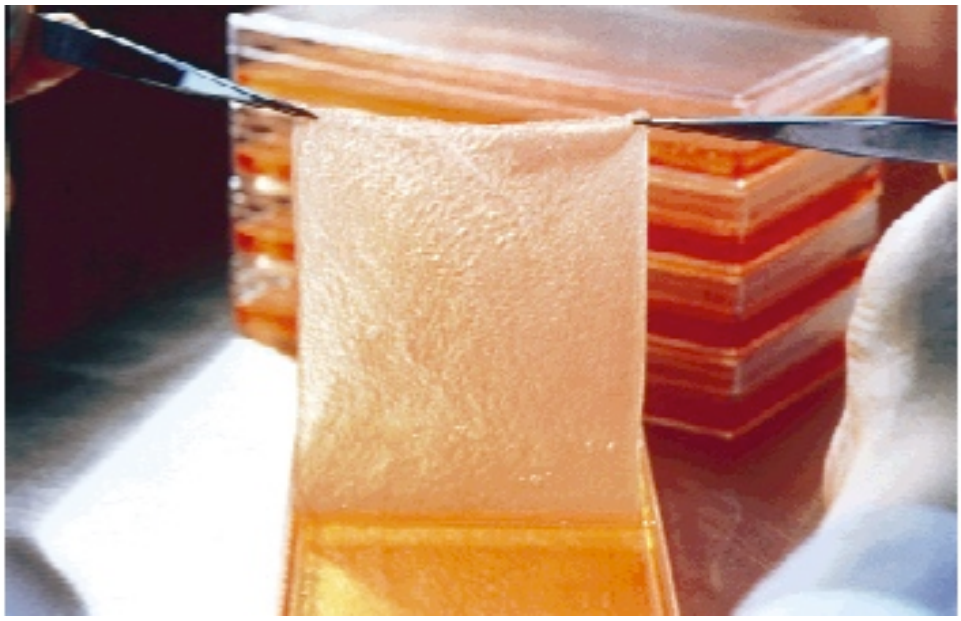


Figure 1.6 Tissue engineered skin [14].

1.2 SCAFFOLDS

1.2.1 Overview

Scaffolds are designed to form an attachable surface for cells and promoting their differentiation and maintenance for the tissue generation from cells. There are certain requirements for scaffolds to be studied with cells for therapeutic purposes. They should be biocompatible and biodegradable [15]. They should be able to mimic the mechanical

properties of the original tissue, they should be porous in structure for nutrients and bioreactive factors to be able to reach the cells and the scaffold also should be able to integrate with the host tissue [16]. There are several appropriate scaffold materials that cells can attach and grow. These can be classified into two as: synthetic scaffold materials and natural scaffold materials [17]. Some of the natural scaffold materials are collagen [18], fibrin [19], alginate [20], hyaluronan [21], silk [22] and chitosan [23-24]. Some of the synthetic scaffold materials are PLA, PGA and PLGA [17].

1.2.2 Synthetic Scaffold Materials

Synthetic polymer materials are preferred in tissue engineering studies because of their ease of control and modification in design and also cheaper non-toxic alternatives for natural scaffold material alternatives (Figure 1.7). One major disadvantage of synthetic materials is that cells cannot recognize them so they should be further modified and they still have the risk of possible rejection after transplantation. Some common synthetic materials and their properties for tissue engineering can be seen in Table 1.1.

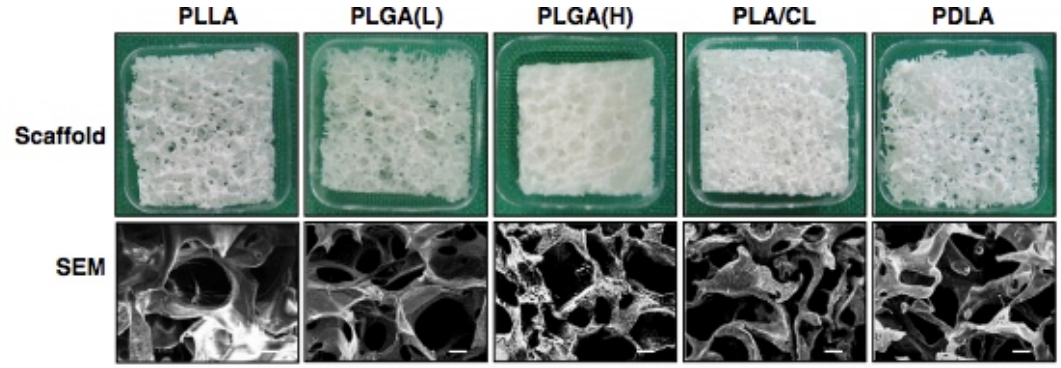


Figure 1.7 Different types of synthetic polymer based scaffolds; rough samples *(on top)* and SEM images *(on bottom)* [25].

Table 1.1 Common synthetic scaffold materials that are used in tissue engineering, adapted from [26].

Material	Biocompatibility	Disadvantages	Application
Poly (lactic acid)	Minimal cytotoxicity, Mild foreign body reaction, Minimal inflammation	Local inflammation, Random chain hydrolysis	Skin; cartilage; bone ligaments; tendons; vessels; nerves; bladder; liver
Poly (glycolic acid)	Minimal cytotoxicity, Mild foreign body reaction, Minimal inflammation	Local inflammation, Random chain hydrolysis	Skin; cartilage; bone ligaments; tendons; vessels; nerves; bladder; liver
Poly (lactic acid-co-glycolic acid)	Minimal cytotoxicity, Mild foreign body reaction, Minimal inflammation	Local inflammation, Random chain hydrolysis	Skin; cartilage; bone ligaments; tendons; vessels; nerves; bladder; liver
Poly (caprolactone)	Minimal cytotoxicity, Mild foreign body reaction, Minimal inflammation	Hydrophobic	Skin; cartilage; bone; ligaments; tendons; vessels; nerves

1.2.3 Natural Scaffold Materials

Natural scaffold materials that are commonly used in tissue engineering are selected because of their similar characteristics with the components of ECM of the cells. This similarity leads cells to a familiar environment that they can attach and grow on these materials. In general ECM is abundant with proteoglycans, glycoproteins, and GAGs so the natural materials that have the same structural properties with these components would have a higher biocompatibility with the applied cells for tissue engineering purposes [26]. Some frequently used synthetic materials and their properties for tissue engineering can be seen in Table 1.2. Natural materials are commonly similar to protein or carbohydrate structures which are compatible with living cells, collagen and fibrin are examples of protein-like materials while chitosan, hyaluronic acid and alginate are examples of carbohydrate-like materials [17, 26].

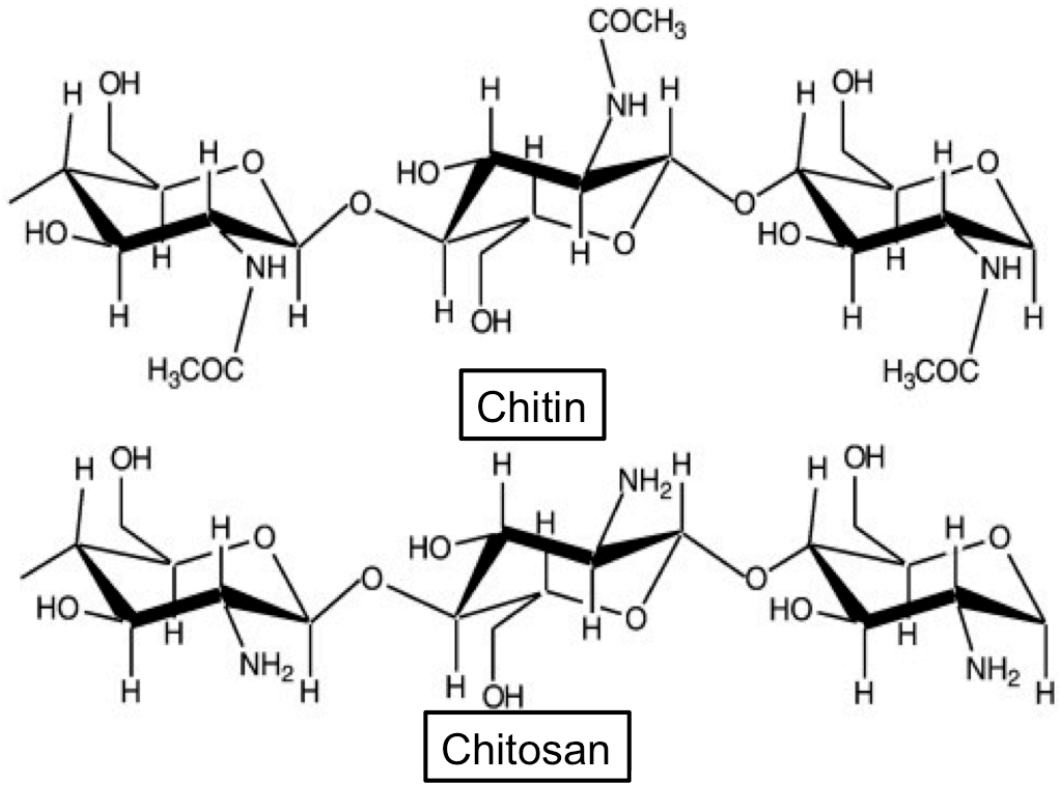


Figure 1.8 Structure of Chitin and its derivative Chitosan [27].

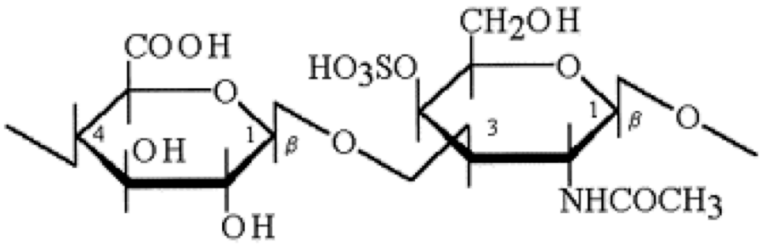
1.2.3.1 Chitosan

One of the most abundant polysaccharides in nature is chitin. It is found on the structure of exoskeleton of arthropods [28-31]. Chitosan is the de-acetylated derivative of chitin (Figure 1.8) [27,31]. The most important aspect of chitosan for tissue engineering applications is that it is similar to the proteoglycans in ECM of various tissues, especially connective tissue (Figure 1.9). This feature of chitosan fulfills the requirement of biocompatibility of the scaffold material for tissue engineering. Some studies also showed that chitosan has antibacterial properties [29-30]. It is reported that chitosan with its polysaccharide structure could be degraded by lysozyme [31-32]. And various implantation applications are made with no toxicity reported [33-35]. Degradation products are reported as hyaluronic acid, chondroitin sulfate, dermatan sulfate, keratin sulfate and glycosylated type-II collagen that are known as non-toxic products as well as some of them can be found in ECM structure of some types of cells [31,36]. Since degradation also depends on the ratio of de-acetylation, chitosan also allows degradation rate to be adjusted with modifications [31].

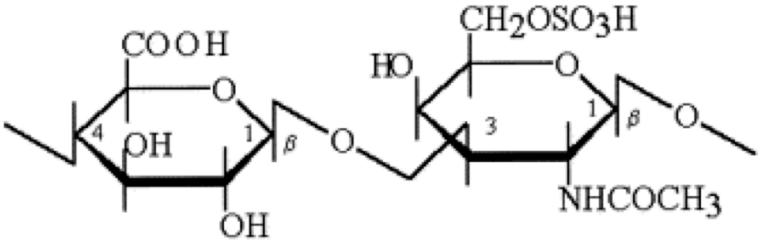
Chitin and fully de-acetylated chitosan are both have maximum crystalline structure capacity. De-acetylation rate is controllable by process. So chitosan is versatile in terms of design. The stiffness of the scaffold and pore size for desired tissue can be regulated. Chitosan itself has a cationic nature and is known to resist in mild pH conditions, but these features also can be adjusted with the design and anionic groups can be attached for release in certain conditions for the desired research purposes [27,31,36-37].

Table 1.2 Common natural scaffold materials that are used in tissue engineering, adapted from [26].

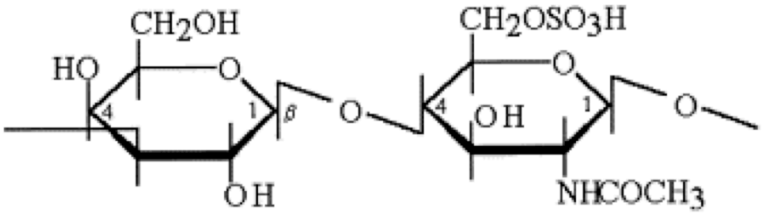
Material	Biocompatibility	Disadvantages	Applications
Collagen	Minimal cytotoxicity, Mild foreign body reaction, Minimal inflammation	Proteoytic removal of small nonhelical telopepties	Skin; cartilage; bone; ligaments; tendons; vessels; nerves; bladder; liver
Hyaluronic acid	Minimal foreign body reaction, No inflammation	Highly viscous solution, Many purification steps after chemical modification	Skin; cartilage; bone; ligaments; nerves; vessels; liver
Alginic acid	Minimal foreign body reaction, No inflammation	Uncontrollable dissolution of hydrogel	Skin; cartilage; bone; nerves; muscle; pancreas
Chitosan	Minimal foreign body reaction, No inflammation	Uncontrollable deacetylation and molecular weight	Skin; cartilage; bone; nerves; vessels; liver; pancreas
Gelatin	Minimal cytotoxicity, Mild foreign body reaction, Minimal inflammation	Weak mechanical property	Skin; bone; cartilage; ligaments; breast
Fibrin	Minimal cytotoxicity, Mild foreign body reaction, Minimal inflammation	Weak mechanical property	Skin, bone, cartilage; liver,tendons; ligaments; vessels
Silk	Highly viscous solution, Many purification steps after chemical modification	Inflammation of sericin	Skin; ligaments; bone; cartilage; tympanic membrane; vessels; tendons



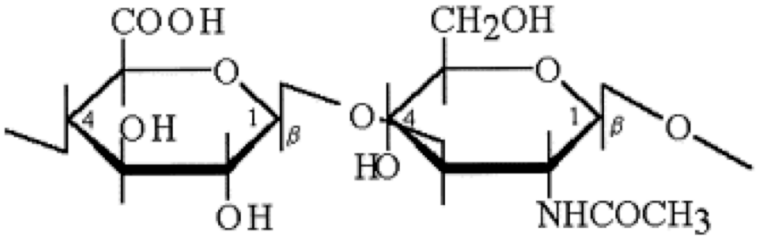
(a) Chondroitin 4-sulfate



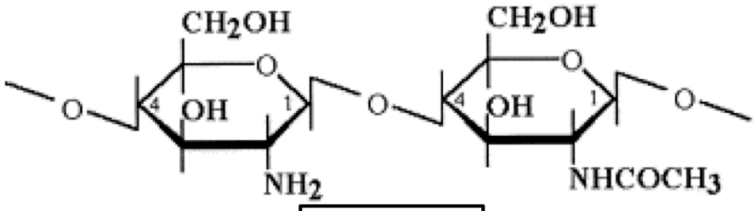
(b) Chondroitin 6-Sulfate



(c) Keratan Sulfate



(d) Hyaluronate



(e) Chitosan

Figure 1.9 Chitosan and similar structures with polysaccharide units: (a) Chondroitin 4-Sulfate (b) Chondroitin 6-Sulfate (c) Keratan Sulfate (d) Hyaluronate and (e) Chitosan [31].

1.2.4 Scaffold Modification

Scaffolds can be modified in order to serve for tissue engineering purpose better and more specific compatibility and function. Composite scaffolds can also be counted as modified scaffolds as some of the components can serve for the tissue environment [38-46]. Also microparticles, nanoparticles, peptides, amino-acid groups and other chemical components can be used for modification [47-49]. PAMAM modification is also one of the recent most used modifications in applications for studies featuring scaffolds.

1.2.4.1 PAMAM Dendrimer Modification/Incorporation

Dendrimers are tree like molecules that usually has a core molecule and around this core, forming a three-dimensional structure like a sphere with its generally symmetrically located branches (Figure 1.10) [50-51]. Polyamidoamine (PAMAM) dendrimers are one of the most known synthetic polymers. PAMAM dendrimers have a ethylenediamine (EDA) core and it can be branched with units of polyamidoamines. Also the core molecule can be designed with ammonia molecule that would have three branching sites whereas EDA would have four branching sites [52-53]. Since terminal group can be modified for desired function, PAMAM is very versatile in structure so it can be used in different biological purposes, like drug delivery and gene delivery [54-59].

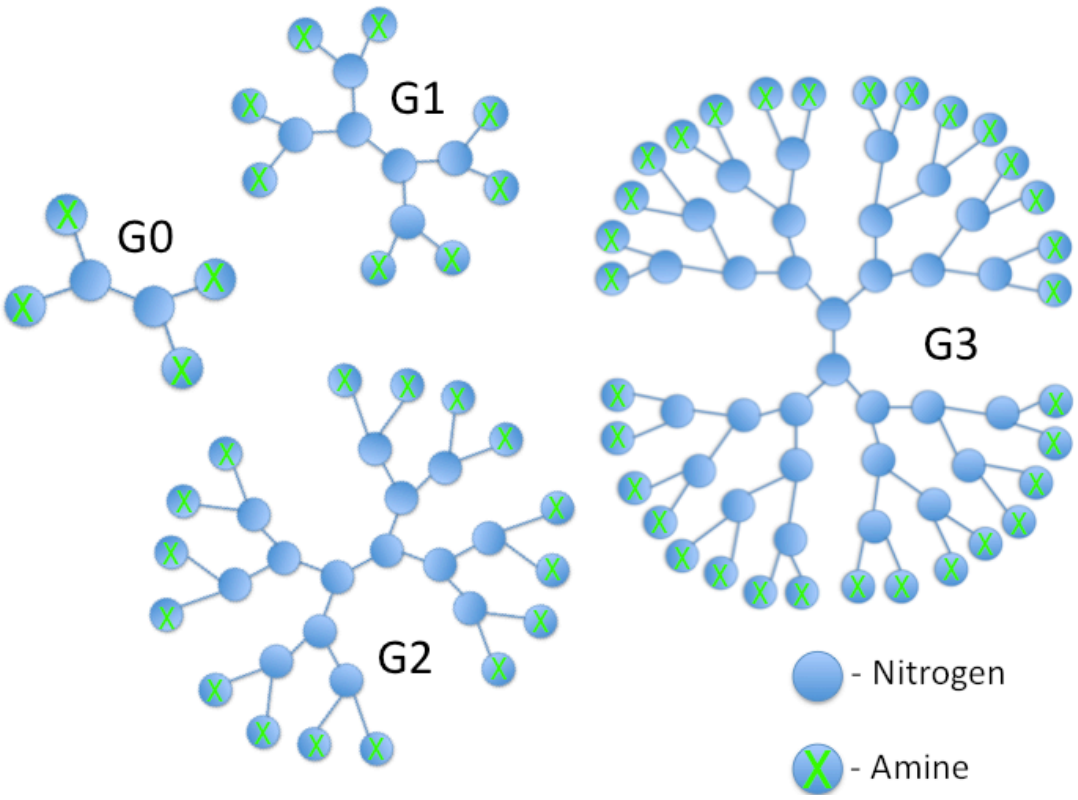


Figure 1.10 Different generations of a dendrimer

1.3 CELL SOURCE

Finding appropriate cell type to study is a major step in the field of tissue engineering. At first cells from the native tissue in the body was used for desired engineered tissues, still it is studied. But with the findings and developments in stem cell biology also brought an alternative cell source to tissue engineering field.

1.3.1 Stem Cells

Stem cells are cells that are capable of self-renewal and differentiation into various types of cells [60-63]. There are two types of stem cells differing in their origin and also in their potency: ES cells and adult stem cells (ASCs). Blastocyst is the source for the ESCs whereas fetal or adult tissue is the source for ASCs [61]. Since there are still controversies for the use of ES cells in ethical aspects, ASCs are the main attraction for the research. ASCs, which include hematopoietic stem cells (HSC) and MSCs, can be obtained from various sources in the body like umbilical cord blood, adipose tissue

and bone marrow [64]. The two types of stem cells are also compared for their potential to differentiate into various lineages of cells so ES cells considered as pluripotent whereas adult stem cells are considered as multipotent in that aspect (Figure 1.11) [65].

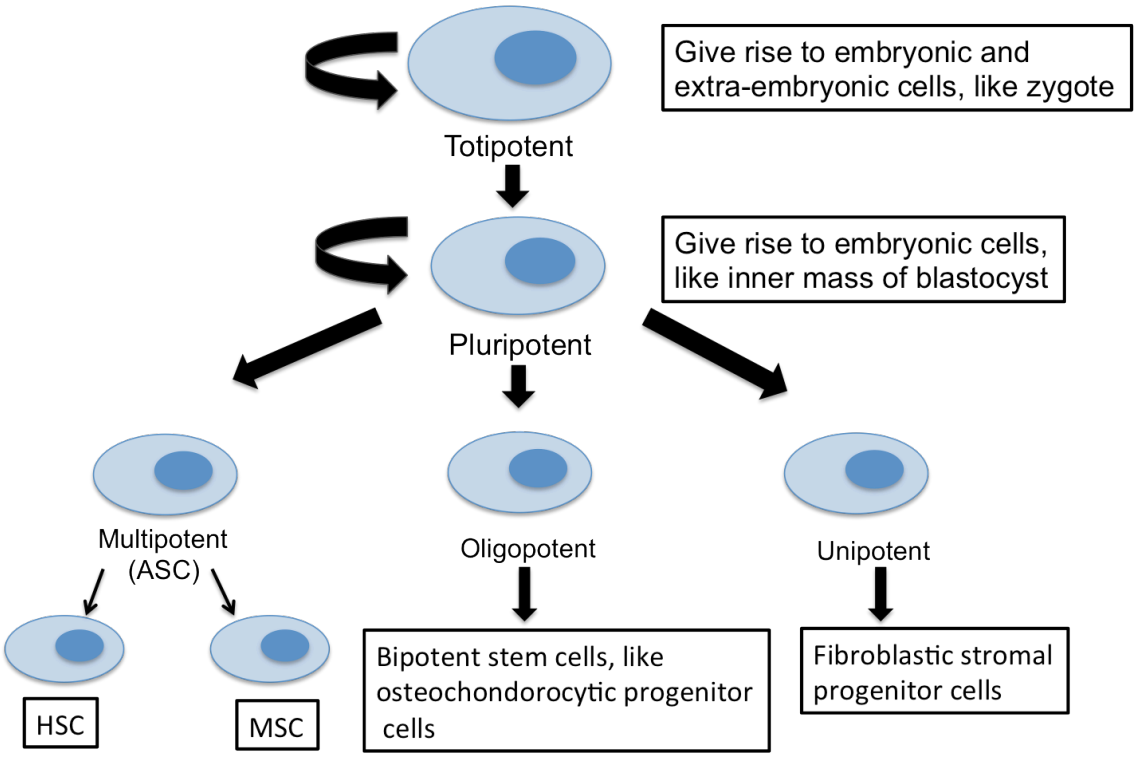


Figure 1.11 Stem cell hierarchy, modified from [65].

1.3.1.1 Mesenchymal Stem Cells

MSCs belong to ASCs and can be derived from various locations in the body starting primarily from bone marrow and then tissues like adipose tissue, fetal tissues, umbilical cord, and dental pulp [66]. Bone-marrow-derived MSCs (BM-MSCs) are known to differentiate into both mesenchymal and non-mesenchymal cells (known as transdifferentiation) [62]. They are shown to differentiate into adipocytes, chondrocytes, osteocytes, stromal cells and neuronal cells in response to right stimuli (Figure 1.12) [62-64]. Taken this information into consideration, MSCs are suitable candidates to study artificial tissue generation for tissue engineering purposes. Also MSCs are easy to obtain from their sources in the body and their self-renewal and proliferation capacity are far better then committed cells [65]. Apart from these, MSCs are shown to have anti-inflammatory and immunosuppressive properties that make them ideal for tissue

engineering studies and this property could enhance the regeneration process which is a plus for this type of studies [67-69].

Mainly MSCs are obtained from bone marrow. After bone marrow sample is taken from a person, the mononucleated cells that are the MSCs in the bone marrow can be isolated with density gradient centrifugation by the use of certain reagents [70]. For an isolated sample of cells to be identified as MSCs, they must qualify some requirements like their cell surface markers. Major important selective cell surface markers for MSCs are reported as positive for CD73, CD90 and CD105 while negative for CD34 and CD45 [60,63-65,69]. This marker selection is achieved by fluorescence-activated cell sorting method (FACS) [70].

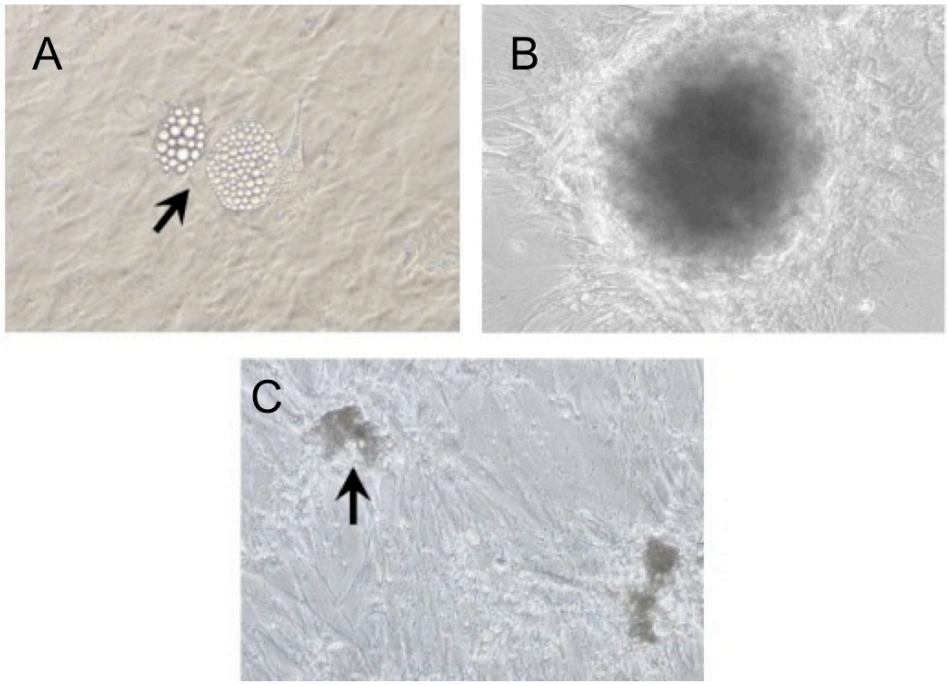


Figure 1.12 hMSCs have capacity to differentiate into different lineages of cells. Adipogenic differentiation of hMSCs (A), chondrogenic differentiation hMSCs (B) and osteogenic differentiation of hMSCs (C), under light microscope, 20X, Fatih University 2011 [63].

1.3.1.1.1 Mesenchymal Stem Cell Line

MSC line describes a family of constantly dividing cells that are produced from a single type of stem cells (Figure 1.13). The source can be a human or animal tissue. It is mainly used for *in vitro* research that requires high concentration of cells to be studied. The stem cells can be immortalized with the technique that utilizes recombinant retroviruses [71-72]. They can proliferate indefinitely and can be used in MSC research related studies [73].

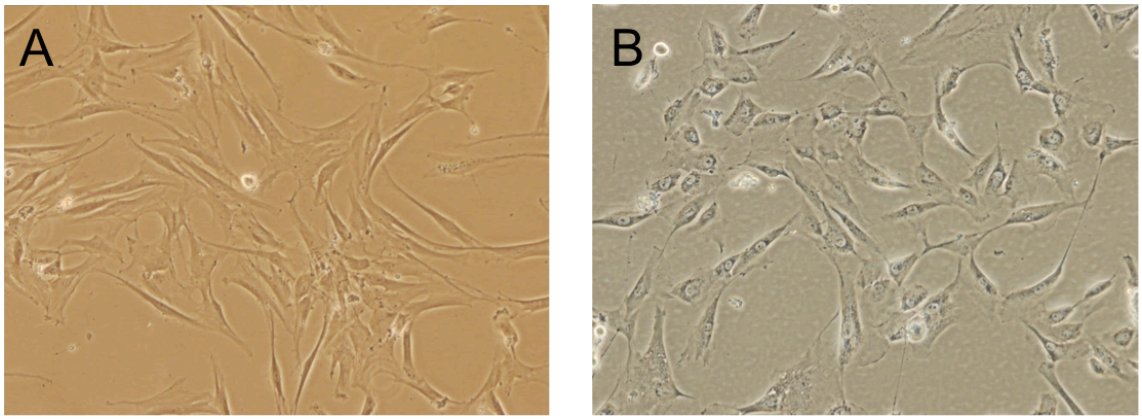


Figure 1.13 Passage 3 bone marrow derived hMSCs (A), passage 4 hMSC line (B), 10X magnification, Fatih University.

1.3.1.2 Other Stem Cell Types

While MSCs are very advantageous compared to other stem cells, still other types of stem cells are also in focus for tissue engineering studies. ES cells are identified as pluripotent with broad differentiation potency but still hard to control the behavior of them once differentiated. Although some countries do not find to work with them ethical, in countries that allows ES cell research it is used in regenerative medicine studies [74]. Other ASC types, like HSCs can be used in limited tissue type generation because of their characteristics [60]. Other than naturally isolated stem cells, recently induced stem cells are also in focus. These stem cells are produced from somatic cells with the overexpression of reported factors in the gene, also called induced pluripotent stem (iPS) cells [75]. There are studies utilizing iPS cells in tissue engineering [76-77].

1.3.2 Other Cell Sources

Somatic cells from the body were the first choice of cell types for tissue engineering studies. According to desired artificial tissue type that is want to be engineered, the cells from the particular tissue could be taken from the living body with clinical methods, and then cultured *in vitro* before using on tissue engineering studies [22,77]. Although still somatic cells from the tissues are being studied, the shortcomings of somatic cells in terms of culturing, proliferation and expansion make them a latter choice in tissue engineering field.

1.4 CARTILAGE

1.4.1 Cartilage Tissue

Cartilage tissue is one of the connective tissue types in humans. It has a massive amount of ECM, which its cells embedded in, when compared others. Its main cell type is called chondrocytes that are stem from chondroblasts, which have mesenchymal origin. Chondrocytes lie in the spaces called lacunae in ECM as groups of two or more. Apart from chondrocytes, fibroblasts are present for producing the ECM components and collagen. And main non-cellular components of ECM of cartilage are, fibers like collagen and elastin and GAGs like hyaluronan, proteoglycans and glycoproteins. These components help cartilage tissue to be resistant to compression, so cartilage tissue is found in the body where tensile strength needed, like knee joints. This tissue is considered flexible yet strong enough to resist compression [78].

There are three types of cartilage tissue differing in the amount of non-cellular components in its ECM. These three types are; hyaline cartilage, elastic cartilage and fibrocartilage (Figure 1.14). Collagen is the only fiber found in hyaline cartilage and this type of cartilage constitutes the embryonic skeleton as well as can be found in joints of long bones [78]. Elastic cartilage contains elastic fibers beside collagen in its ECM and can be found in structures like ear and epiglottis. Fibrocartilage is similar but known less firm than hyaline cartilage containing thick collagen components in it ECM, it is found in knee joint and between intervertebral discs [78].

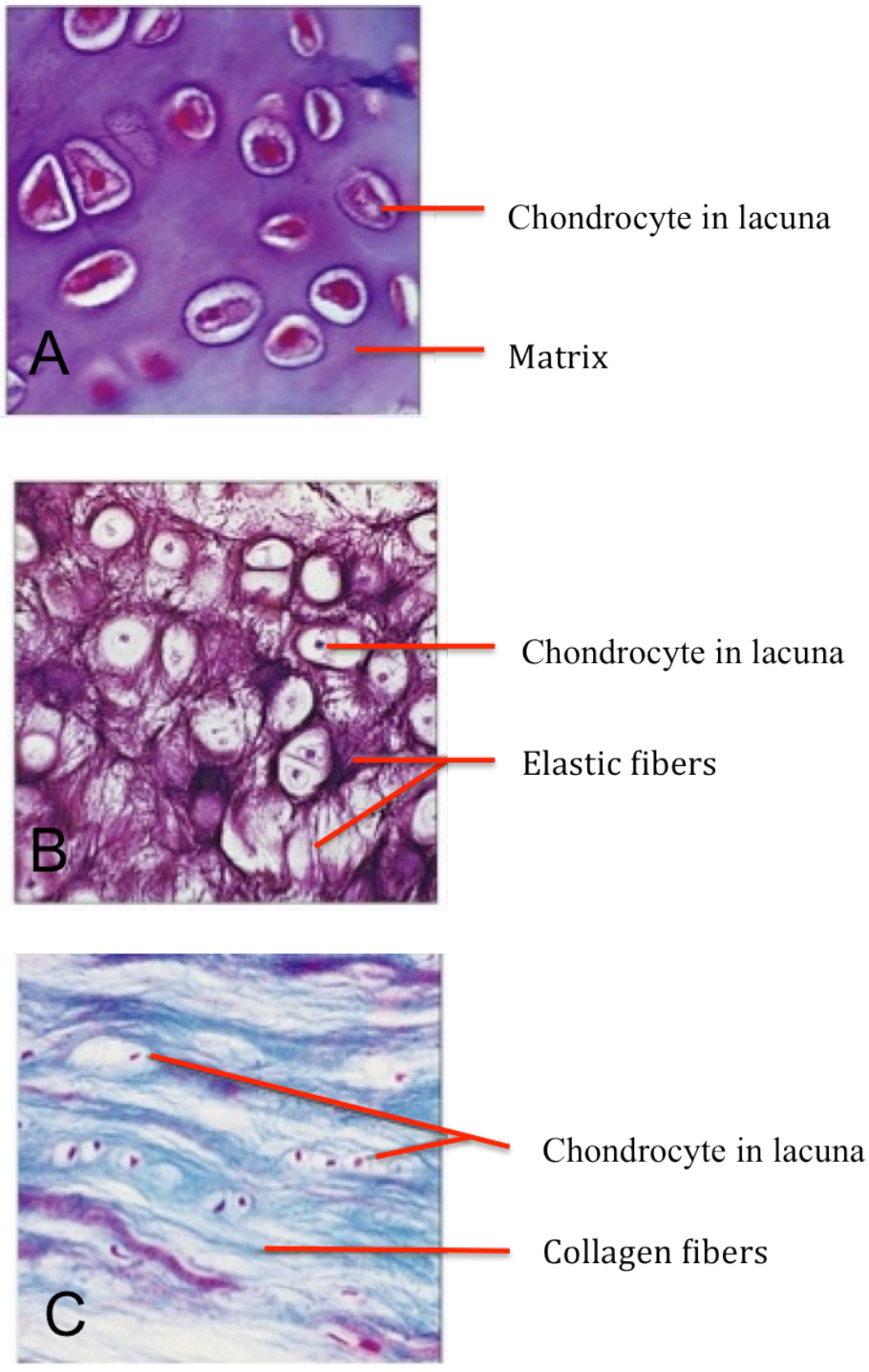


Figure 1.14 Cartilage tissue types: hyaline cartilage from trachea (A), elastic cartilage from ear (B), fibrocartilage from intervertebral disc (C) [78].

CHAPTER 2

MATERIALS AND METHODS

2.1 CHITOSAN BASED SCAFFOLD SYNTHESIS, DESIGN AND CHARACTERIZATION

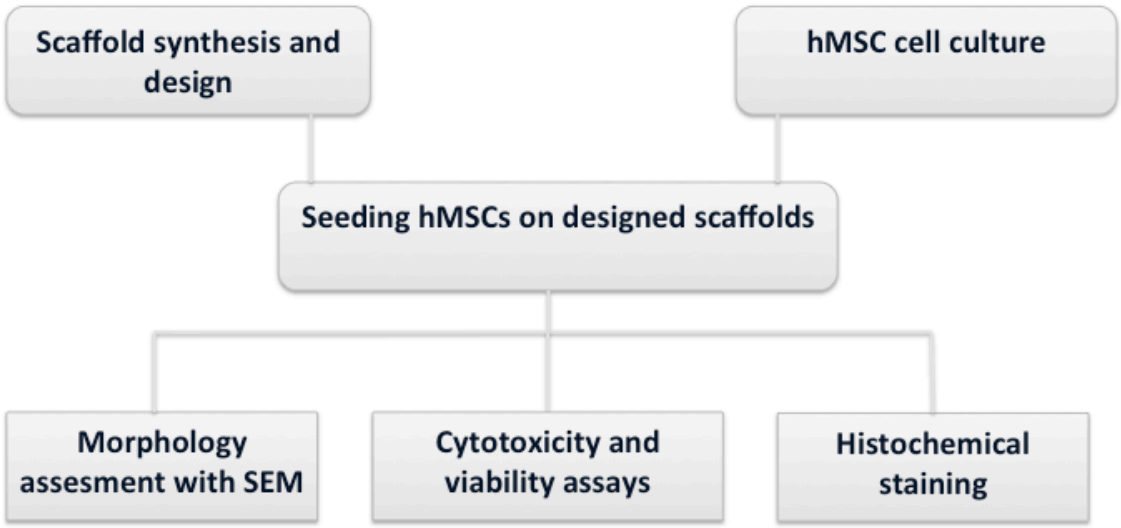


Figure 2.1 Experimental design diagram.

2.1.1 Preparation of Chitosan Based Scaffolds

Scaffolds were prepared with using high viscosity chitosan. five types of dendrimer incorporated chitosan based scaffolds were synthesized with different levels of PAMAM incorporation: Chitosan (with no PAMAM, CTS), Generation 0 (G0), Generation 1 (G1), Generation 2 (G2), Generation 3 (G3). For chitosan scaffold, high

viscosity chitosan (2%) was dissolved in 1% acetic acid solution using magnetic stirrer. For G0, after 2% high viscosity chitosan was homogenized, 1% amine functional 1st generation PAMAM dendrimer was incorporated on chitosan. For G1, after 2% high viscosity chitosan was homogenized, 1% amine functional 2nd generation PAMAM dendrimer was incorporated on chitosan. For G2, after 2% high viscosity chitosan was homogenized, 1% amine functional 3rd generation PAMAM dendrimer was incorporated on chitosan. For G3, after 2% high viscosity chitosan was homogenized, 1% amine functional 4th generation PAMAM dendrimer was incorporated on chitosan. After the solution was homogenized, for each type of solution, 1.5 mL of 0.25% of glutaraldehyde was added to the mixture and stirred for 30 minutes until the reaction was completed. Then the mixture was transferred into a mold (or a syringe) and pre-freezed at -20°C for 24 hours. The frozen mixture was freeze-dried at -80°C for 24 hours to obtain the scaffolds. Then the scaffolds were neutralized by 2% NaOH and 5% NaBr for 2 hours and further washed with de-ionised water. Finally, the scaffolds were freeze-dried and stored at -20°C for further use.

2.1.2 Characterization

2.1.2.1 Swelling Studies

The swelling studies were performed in PBS (phosphate based saline) at pH 7.4 at 37°C. The dry weight of the scaffold was measured and noted as Wo. Scaffolds were placed in PBS buffer solution for 1 week at pH 7.4. Scaffolds were removed each day, filter paper was used for removing the adsorbed water on the surface and wet weight of the scaffolds was recorded as Ww. The ratio of swelling was determined using the formula:

$$Swelling\ ratio = (Ww - Wo) / Wo \quad (2.1)$$

2.1.2.2 Water Uptake Abilities

Dry scaffolds were weighed (Wdry) and immersed in distilled water for 24 hours. After 24 hours, scaffolds were gently removed from the beaker and placed on a wire mesh rack. Excess water was drained and scaffolds were weighed (Wwet) after 5 minutes to determine water uptake.

The percentage of water absorption (EA) of the scaffolds at equilibrium were calculated using following equation:

$$EA = (Wwet - Wdry) / Wdry \times 100 \quad (2.2)$$

2.1.2.3 Porosity Measurement

The total porosity was determined by the liquid displacement method. A scaffold with the weight *W* was immersed in a graduated cylinder containing the known volume *V1* of ethanol. The cylinder was placed in vacuum to force the immersion of the ethanol into the pores of the scaffold until no air bubble emerged from the scaffold. The total volume of the ethanol and scaffold was then recorded as *V2*. The volume difference *(V2- V1)*, was the volume of the skeleton of the scaffold. The scaffold was removed from the ethanol and the remaining ethanol volume was measured as *V3*. The total volume of the scaffold, *V*, was determined then:

$$V = V2 - V3 \quad (2.3)$$

2.1.3 *In vitro Degradation of Scaffolds*

All the scaffolds were treated with Lysosyme enzyme for biodegradability. For 4 weeks, scaffolds, in duplicate, were treated with 500 μg/ml lysozyme in PBS at 37°C. Before lysozyme treatment all the scaffold samples were weighed and initial weight of each samples were recorded as initial weight (*W1*). At the end of each week, scaffold samples were rinsed with ddH_2O, and then freezed in small amount water at -20°C then lyophilized. After lyophilization, scaffolds were weighed for the final weight (*W2*). Weight loss for each scaffold was calculated:

$$Weight\ Loss\ (\%) = (W2-W1/W1) \times 100 \quad (2.4)$$

2.2 CELL CULTURE

Immortalized UE7T-13 cells that obtained from Riken Bioresource Center, Japan. UE7T-13 cells are human bone marrow derived, fibroblast like MSCs that were immortalized with recombinant retroviruses expressing the E7 and hTERT.

2.2.1 Seeding and Subculture of hMSC Line

After seeded into culture flasks, cells were attached to the surface and proliferated to form colonies. When cells in the flask became 80% confluent in the flask surface, they were subcultured. DMEM (Dulbecco's Modified Eagle Medium), FBS (Fetal Bovine Serum), PBS and trypsin for subculture were warmed in 37°C water bath before protocol.

After seeding cells into culture flasks, cells were attached to the flask surface and colonies were started to form. When cells became 80% confluent they were subcultured. Before the subculture, DMEM, FBS, PBS and trypsin were warmed to 37°C in water bath. Conditioned medium in the flask was collected to a centrifuge tube by a sterile pipette and then the flask surface was washed with 5 ml of PBS (Biochrome). After PBS was discarded, 3 ml of pre-warmed 0.25% Trypsin/EDTA (SIGMA) was added to the flask and kept at 37°C incubator for 5 minutes at most. Then cells were observed under invert light microscope for their detachment from the flask surface. When detachment was confirmed, 2 ml of FBS added to the flask to inactivate the trypsin activity. The cells in the flask with trypsin and FBS was transferred into new 15 ml centrifuge tube and centrifuged at 1850 rpm for 5 minutes at room temperature. Supernatant was removed by leaving about 0.5 ml of the cell suspension at the bottom. Pellet was finger mixed and volume was completed to 10 ml with DMEM in order to wash any remaining trypsin from suspension. The suspension was centrifuged one more time. Supernatant discarded and pellet was suspended in 2 ml DMEM. Cells were counted by hemocytometer. After counting, hMSCs were seeded with a cell concentration of 1500 cells/cm^2 with 10% FBS containing DMEM for expansion. Medium was refreshed once between two subcultures if required. Subculture of cells was repeated in every 3-4 days.

2.2.2 Freezing and Thawing hMSC Line Cells

Standard subculture protocol was applied and cells were counted with hemocytometer. The suspension was centrifuged to form a pellet of cells. After discarding supernatant completely and carefully, cells were resuspended in FBS to form a cell concentration of $1x10^6$ cells/ml. Cryovial tubes were placed on ice box and cell suspension was transferred to each tube as 900 µl per tube. 100 µl Dimethyl Sulfoxide

(DMSO, Applichem) was added into each tube drop by drop as mixing the DMSO into suspension by swirling the tip of pipette in each drop. Cryovial tubes were placed at -20°C for 1 hour and then replaced into -80°C and kept in for overnight. For long-term storage, the next day cryovial tubes were transferred into liquid nitrogen tank (-196 °C).

Frozen cells were stored to be thawed and used in experiments. Before thawing, 10 ml of pre-warmed DMEM to 37 °C was added into a 15 ml centrifuge tube. Then cryovial tube of desired cells were taken from the nitrogen tank and warmed to 37°C in water bath. Thawed cell suspension in the cryovial tube was transferred quickly to the centrifuge tube containing pre-warmed DMEM and centrifuged at 1850 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 10 ml medium for second wash in order to eliminate remaining DMSO. Suspension centrifuged on more time. All of the cells in the pellet were seeded with 20% FBS containing DMEM and the medium was refreshed next day, to discard the any dead cells.

2.3 ATTACHMENT OF hMSC CELLS TO CHITOSAN SCAFFOLDS

2.3.1 Seeding hMSCs onto Chitosan Scaffolds

Standard subculture protocol was applied and cells were counted with hemocytometer. Cells were centrifuged to form pellet and after removing supernatant, cells were suspended for desired cell concentration, about $20x10^6$ cells/ml. 50 μl cell suspension was dropped carefully onto each scaffold (~2 mm) that were placed in 24-well plate. The scaffolds were incubated at 37°C for 4 hours for attachment of cells onto scaffolds. Then 1 ml 20% FBS containing DMEM was added into each well. Each scaffold was studied in double.

2.3.2 Attachment Assessment of hMSCs on Chitosan Scaffolds

To assess the number of attached hMSCs to chitosan-based scaffolds, non-attached cell number was calculated. After at most 4 hours incubation of hMSCs on chitosan-based scaffolds on 24-well plate, scaffolds were removed to a new 24-well plate while DMEM in the plate was collected into 15 ml centrifuge tube. Then the scaffolds were squeezed for collecting excess DMEM and washed with PBS for collecting any non-attached cells. Then the old plate was trypsinized and the trypsin was

neutralized with FBS and collected into tubes. All collected solutions were centrifuged at 2000 rpm for 5 minutes. Supernatant was removed and pellet was resuspended in 500 μl solution. Then any non-attached cells in the solution were calculated by mixing 20 μl resuspended solution with 20 μl Tryphan blue dye by using hemocytometer.

2.4 SCANNING ELECTRON MICROSCOPY (SEM) IMAGING OF CHITOSAN SCAFFOLDS

Synthesized and neutralized chitosan based scaffolds were further sterilized under UV for 40 minutes. Rough samples were coated with a layer of gold-palladium mixture with EMITECH K550X plasma coater. Imaging was performed at 10kV using SEM (JCM-5000 NeoScope, JEOL) to reveal scaffold morphology. Imaging performed in BINATAM, Fatih University.

2.5 PROLIFERATION AND CYTOTOXICITY EXPERIMENTS

2.5.1 WST-1 Cell Proliferation Experiment

hMSC line was seeded ($25x10^4$) onto 5 types of scaffolds (1-1.5 mm) in triplicate to 96-well plates in 150 μl DMEM with 20% FBS and let incubation for 24 hours before the experiment. Cell proliferation assay was performed with the WST-1 reagent (ROCHE) for each type of scaffold (CTS, G0, G1, G2, G3). Proliferation Assay was done according to the manufacture's instructions. Afterwards absorbance was measured at 450 nm using an ELISA reader.

2.5.2 Lactate Dehydrogenase (LDH) Cytotoxicity Experiment

hMSC line was seeded ($100x10^4$) onto 5 types of scaffolds (1-1.5 mm) in triplicate to 48-well plates in 750 μl DMEM with 20% FBS and let incubation for 24 hours before the experiment. Cytotoxicities were determined with the lactate dehydrogenase (LDH) leakage assay (ROCHE) for each type of scaffold (CTS, G0, G1, G2, G3). Cytotoxicity assay was performed according to the manufacture's instructions. Afterwards, absorbance was measured at 490 nm using the ELISA reader.

2.6 CHONDROGENIC DIFFERENTIATION OF hMSCs

2.6.1 Chondrogenic Differentiation of hMSCs

For chondrogenic differentiation, hMSC line was harvested at P5 by trypsinization and seeded onto 24-well plate in duplicates with a concentration of $8x10^5$ cells/scaffold as described earlier. Next day, medium was changed into chondrogenic differentiation. Pre-warmed Stempro Chondrocyte Differentiation Basal Medium (Gibco) containing 10% Stempro Chondrogenesis Supplement (Gibco) was used. The medium was changed every 3 days. After 21 days of cultivation, scaffolds were ready for Alcian Blue staining and RNA isolation experiments.

2.6.2 Alcian Blue Staining of Chondrogenically Differentiated hMSCs

3% acetic acid solution was prepared with distilled water to solve Alcian Blue dye. 1% Alcian Blue solution was prepared with 3% acetic acid solution, stirred overnight for dye to be completely dissolved. Differentiated cells were washed with PBS and fixed with 4% paraformaldehyde for fixation beforehand. Alcian Blue was applied to fixed cells for 40-50 minutes. The dye was then removed and scaffolds were air-dried. Photos of staining are taken under light microscope.

CHAPTER 3

RESULTS

3.1 UE7T-13 hMSC LINE CULTURE

hMSC line that obtained from Riken Bioresource Center, Japan was cultivated *in vitro*. hMSC line was observed to be smaller in size when compared to primer hMSCs that obtained from bone marrow (Figure 3.1). hMSC line was subcultured in every 3-4 days with DMEM (10% FBS).

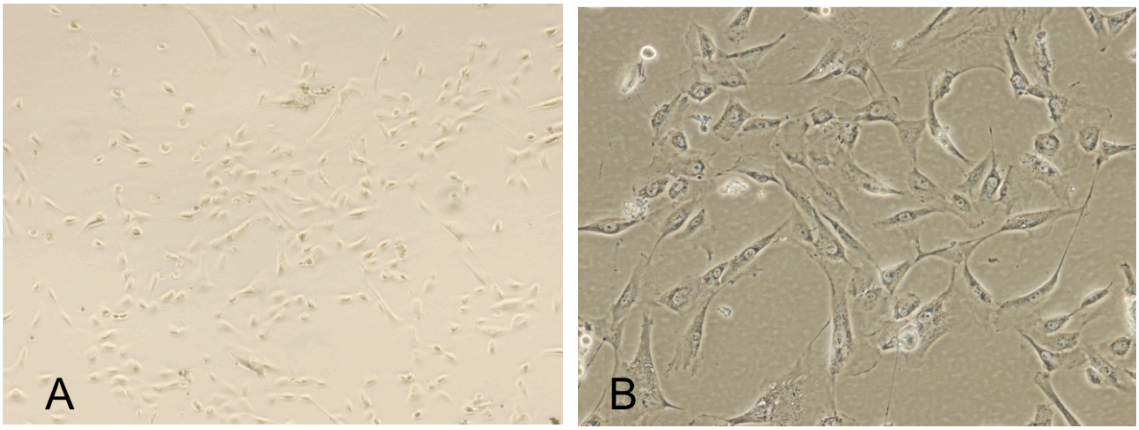


Figure 3.1 Passage 4 hMSC line under light microscope, 4X (A) and 10X (B) magnification, Fatih University.

3.2 SEM IMAGING

Rough non-seeded scaffolds and hMSC line seeded scaffolds were photographed under SEM. For SEM imaging all samples with different PAMAM generations were used.

The SEM images at different magnifications showed that all the chitosan scaffolds have very smooth surface and very porous in structure. The pores are large enough to adsorb any medium or liquid and also for cells to move inside and to attach, Figure 3.3 - Figure 3.7. The scaffolds were prepared with high viscosity solutions 2% in concentration.

From chitosan to G3 generation PAMAM incorporated chitosan scaffold slight decreasing in pore size was observed. This would due to more branching of PAMAM in G3 generation compared to chitosan with no PAMAM in structure. The slight decrease in the size of the pores had no effect for the desired size that enables for the movement and attachment of cells, as can be seen by comparing Figure 3.3 and Figure 3.7.

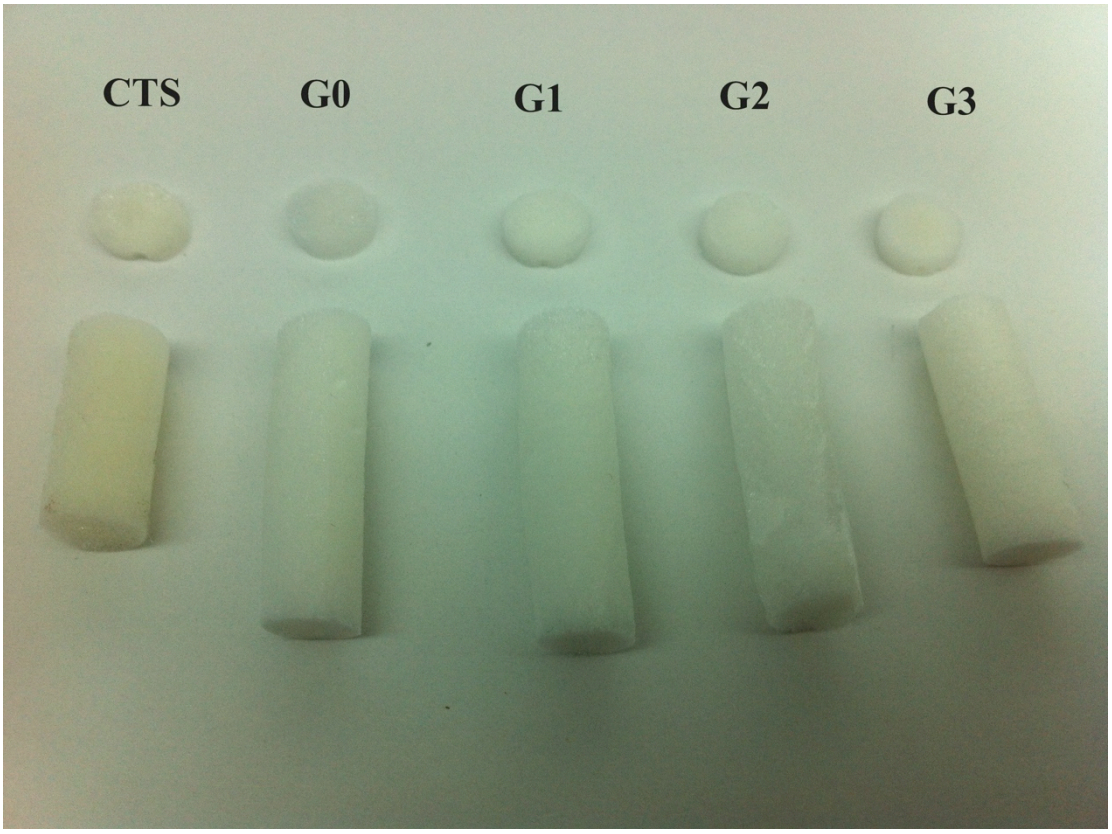


Figure 3.2 PAMAM incorporated chitosan-based scaffolds dry and rough samples, Şenel Lab, Fatih University.

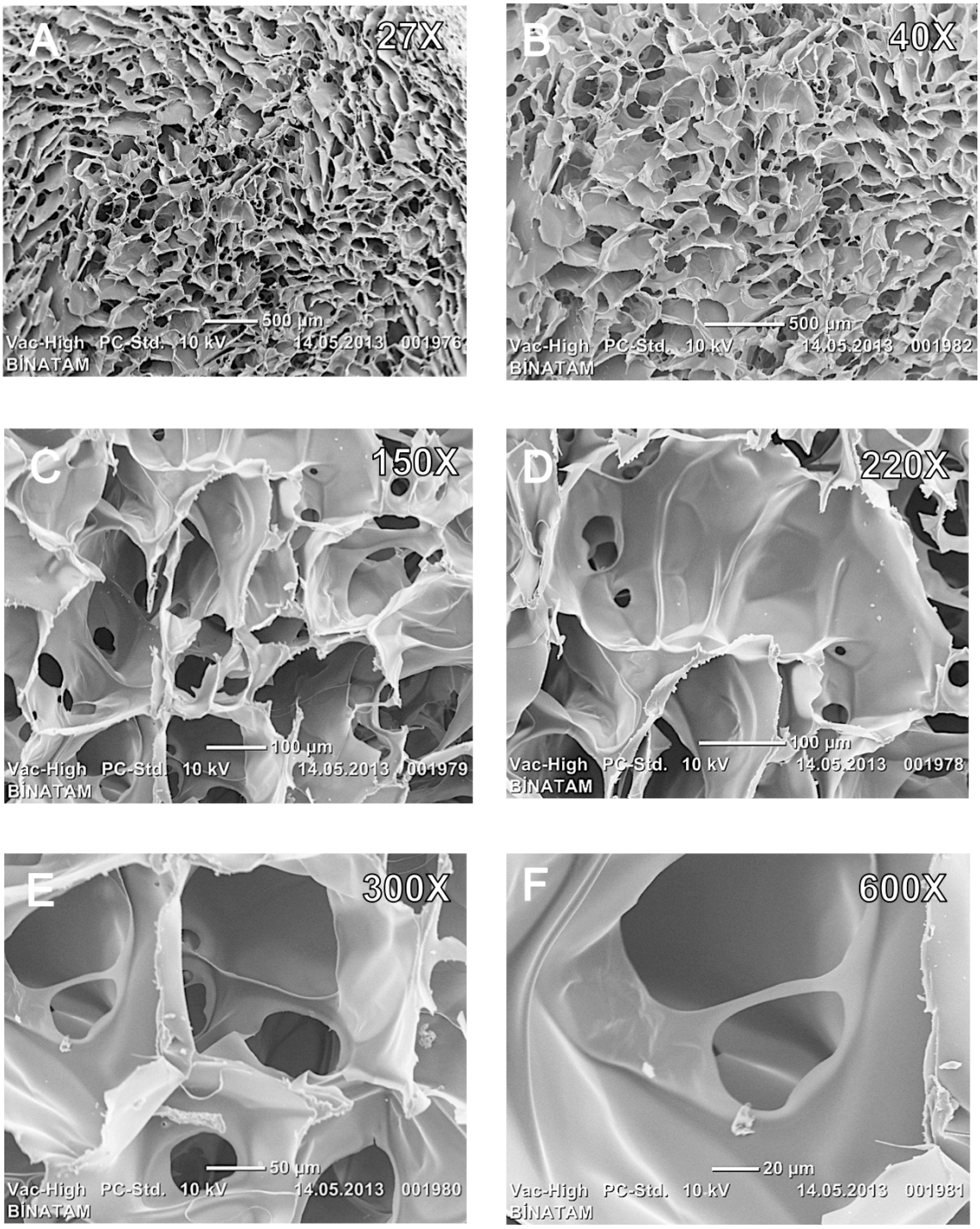


Figure 3.3 SEM images of CTS scaffold *(A-F)*.

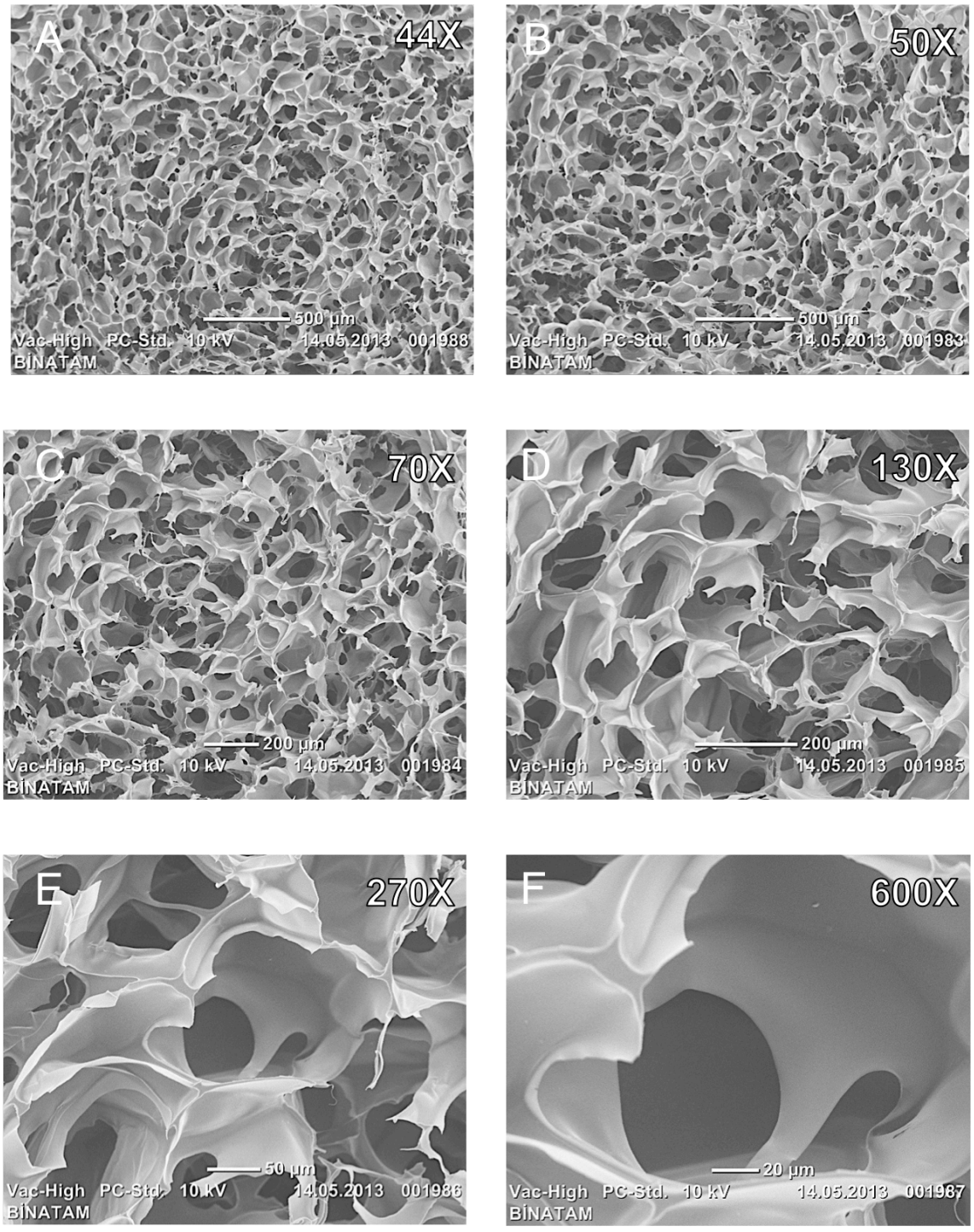


Figure 3.4 SEM images of G0 PAMAM incorporated chitosan scaffold *(A-F)*.

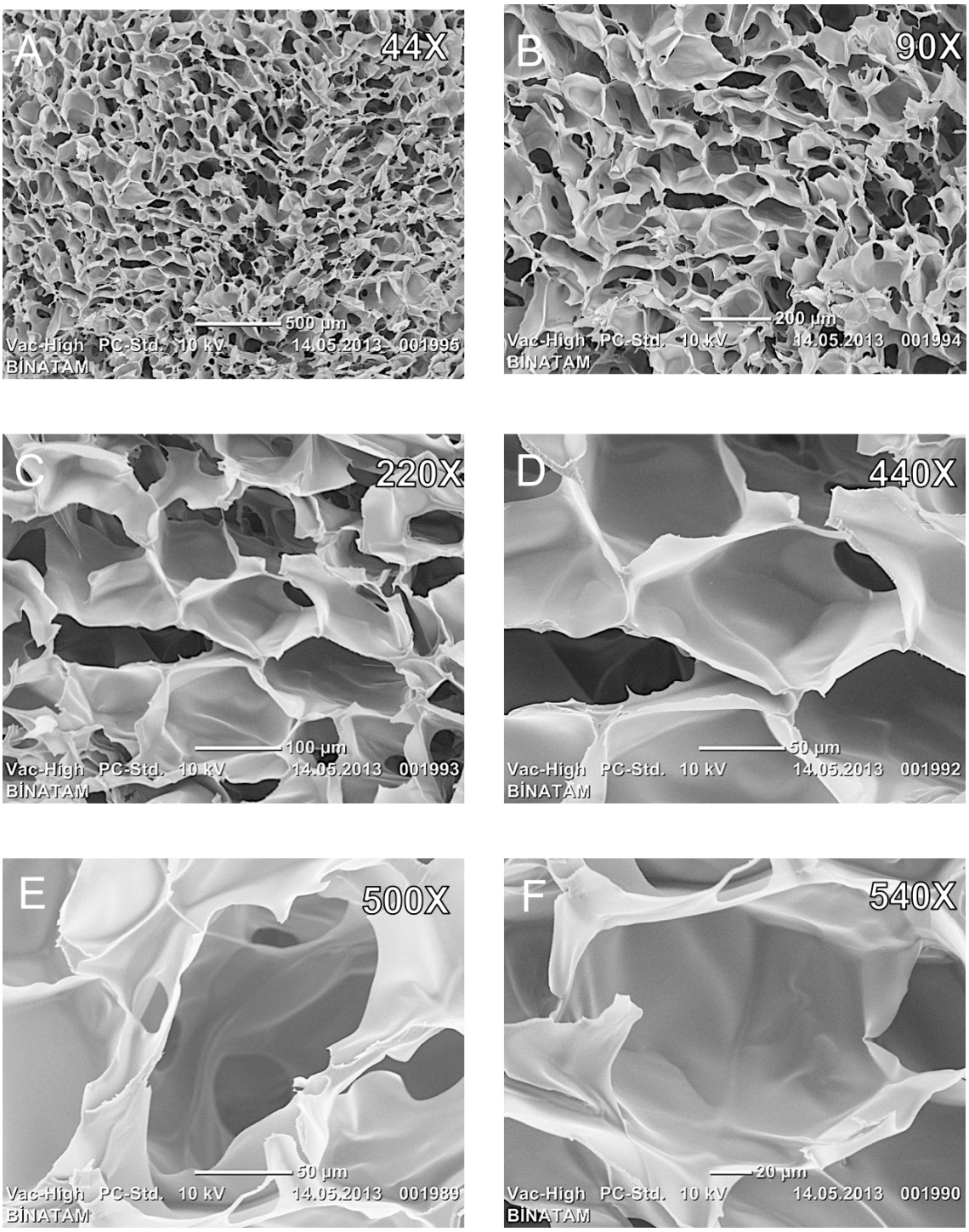


Figure 3.5 SEM images of G1 PAMAM incorporated chitosan scaffold *(A-F)*.

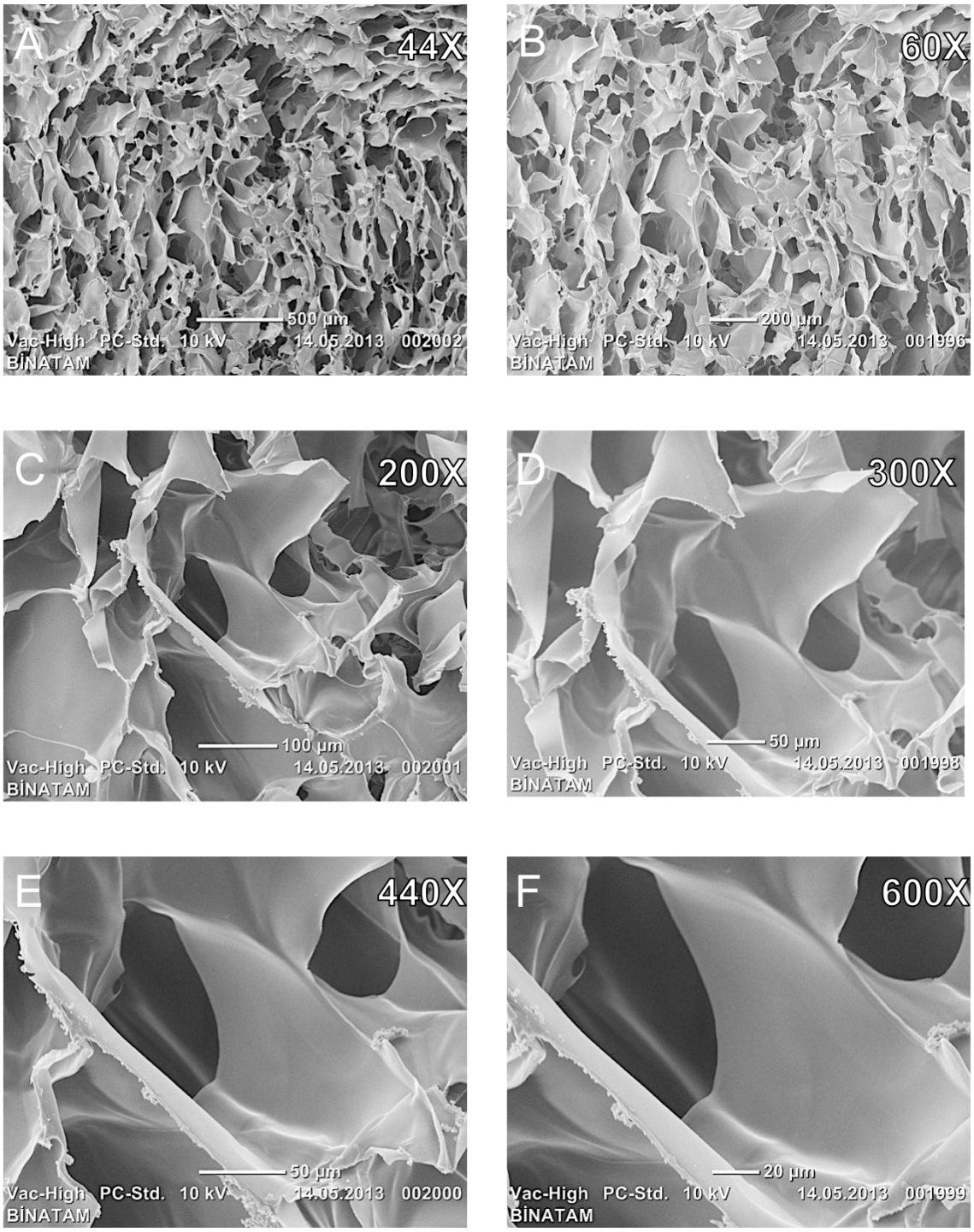


Figure 3.6 SEM images of G2 PAMAM incorporated chitosan scaffolds *(A-F)*.

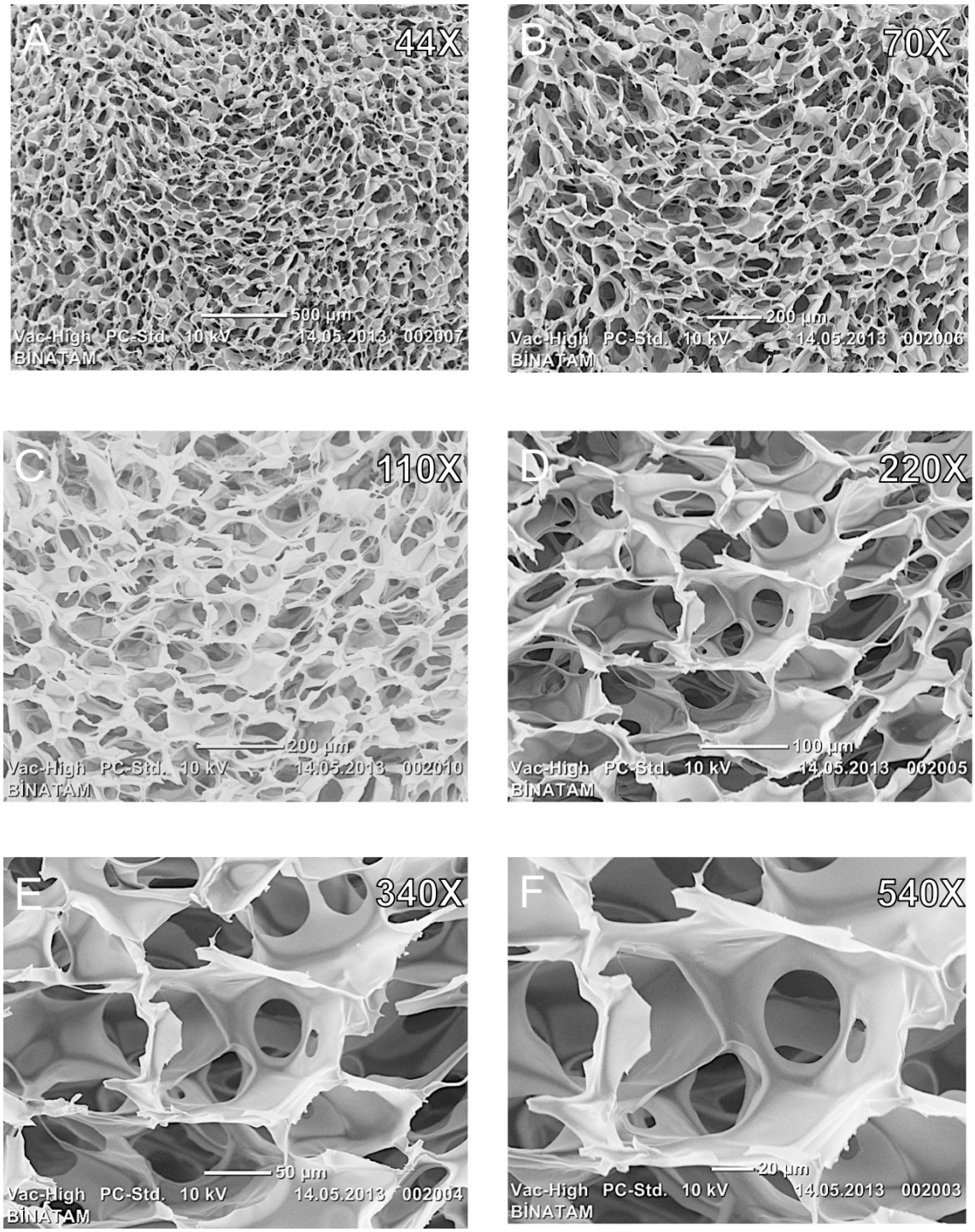


Figure 3.7 SEM images of G3 PAMAM incorporated chitosan scaffolds *(A-F)*.

3.3 POROSITY, WATER UPTAKE AND SWELLING TESTS

The scaffolds were examined by their porosity, swelling and water uptake capabilities. All types of scaffolds showed similar characteristics for these tests (Table 3.1). All types of scaffolds showed about 95-97% porosity in their structure. All types of scaffolds showed that they could absorb water up to 25% of their initial weights. Also all types of scaffols showed that they could swell up to 30% of their initial sizes.

Table 3.1 Characterization results of scaffolds.

Scaffold Type	*Porosity (%)*	*Water Uptake*	*Swelling*
CTS	97.4 ± 0.45	24.9 ± 1.73	29.3 ± 1.55
G0	96.8 ± 0.25	24.5 ± 3.16	27.5 ± 2.33
G1	96.6 ± 0.29	22.9 ± 2.31	26.7 ±7.78
G2	96.2 ± 0.24	26.2 ± 3.80	27.9 ± 2.76
G3	95.8 ± 0.29	24.8 ± 2.00	27.4 ± 2.70

3.4 *IN VITRO* DEGRADATION TEST

Lysosyme treatment was used to test *in vitro* degradation of chitosan-based scaffolds. 5% solution of Lysosyme enzyme in PBS was used for four weeks for assessment of degradation. Weight loss of chitosan and PAMAM incorporated scaffolds were calculated as percentage of their initial weights.

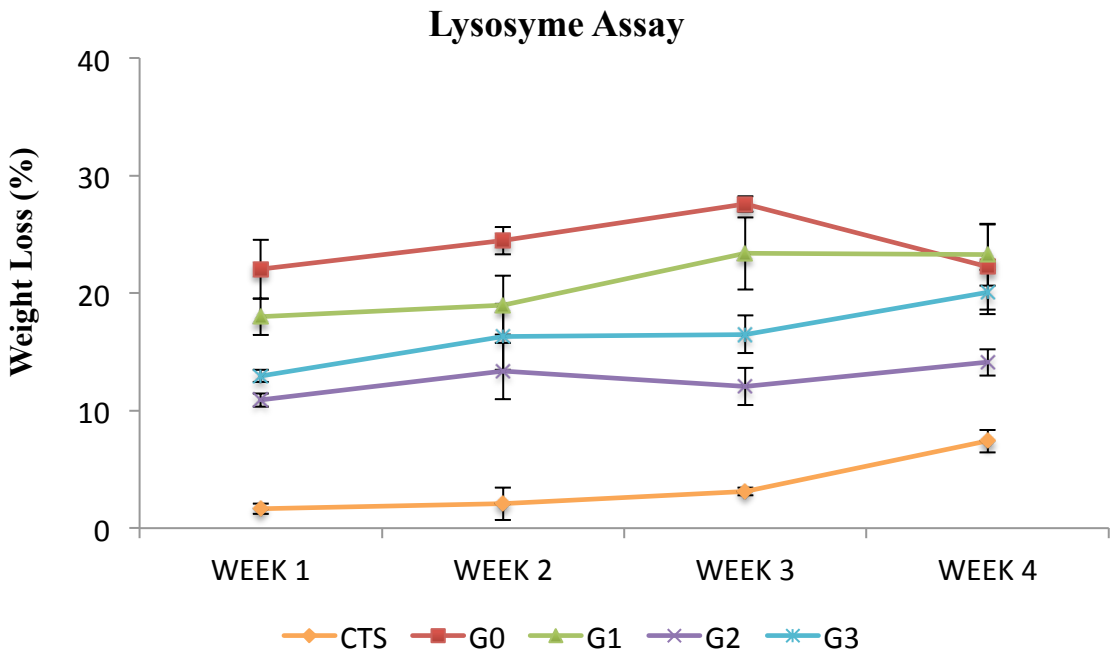


Figure 3.8 Percentage weight loss of scaffolds after lysozyme treatment.

All types of scaffolds showed weight loss after four weeks of treatment with lysosyme. At the end of four weeks, chitosan without PAMAM showed the least weight loss as up to 10%, maximum (Figure 3.8). The most branched, G3 PAMAM incorporated chitosan scaffold showed average loss of all the scaffold types with weight loss up to 20% at the end of four weeks (Figure 3.8).

3.5 ATTACHMENT OF hMSC LINE ON CHITOSAN SCAFFOLDS

Initial cell attachment onto chitosan-based scaffolds was determined with simple cell counting under light microscope. The hMSC-seeded scaffolds were handled after four hours of incubation post-seeding. The medium that the scaffolds were cultured and the washing solutions were all collected and centrifuged for any non-attached cells. Percentage of initial attachment was calculated by counting non-attached cells. According to data all scaffolds showed 75% and more initial attachment to chitosan scaffolds (Figure 3.9).

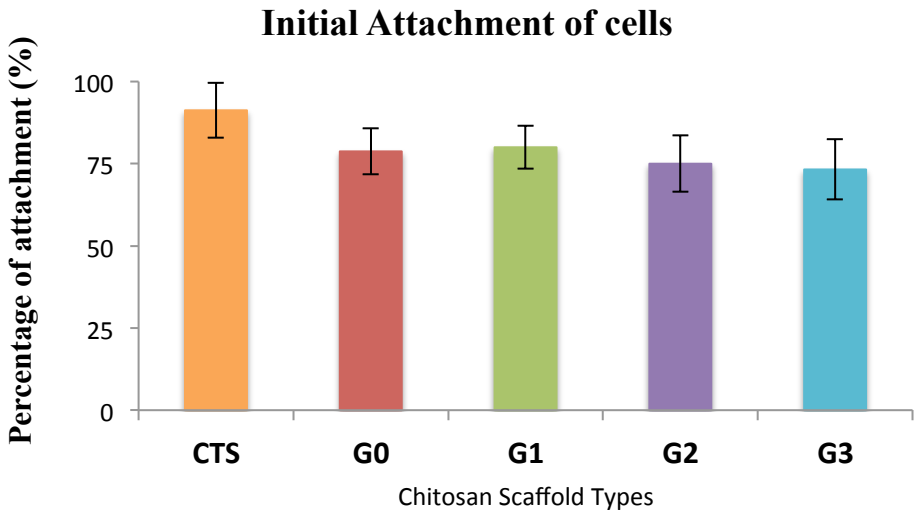


Figure 3.9 Percentage of attached hMSCs onto chitosan and PAMAM incorporated scaffolds.

3.6 DAPI STAINING

To confirm that the cells can attach the scaffold surface, DAPI staining procedure was used. DAPI staining of the scaffolds showed that seeded cells were present inside the scaffolds. Under observation of fluorescence microscope, seeding cell density seemed independent from the number of cells that were seeded initially. Under light microscope $5x10^4$ and $10x10^4$ cells seeded scaffold showed similar density of nuclei with DAPI staining (Figure 3.10).

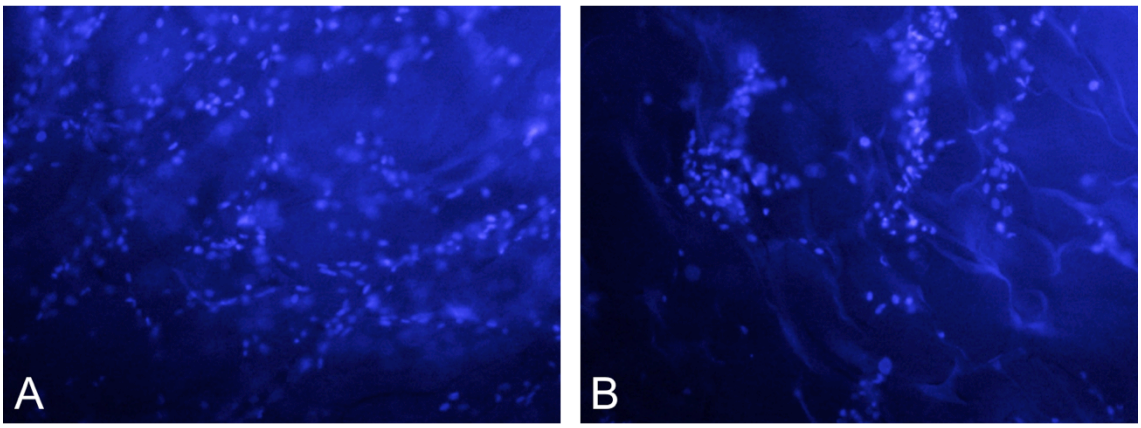


Figure 3.10 DAPI staining of hMSC line seeded on non-incorporated chitosan scaffold, $5x10^4$ cells (A) and $10x10^4$ cells (B), at day 12, under fluorescence microscope, 10X magnification.

3.7 CELL VIABILITY AND CYTOTOXICITY

3.7.1 WST-1 Cell Proliferation Assay

WST-1 is a cell proliferation/cell viability assay that measures the enzymatic reaction of tetrazolium salts to be cleaved into formazan by cellular enzymes that are secreted by mitochondria of living cells (Figure 3.11). So if the living cells are abundant in the culture, cleavage into formazan by the cells will be more rapid. This can be understood by the color of reaction since formazan makes the medium solution to be seen dark red in color. Also can be understood by spectrophotometry.

The control hMSCs seeded on tissue culture plates showed almost linear and increasing proliferation with WST-1 reagent (Figure 3.12). When proliferation rate of hMSCs on scaffolds were analyzed, chitosan and G3 PAMAM incorporated scaffold types showed more significant fold values (Figure 3.13 and Figure 3.17) than G0, G1 and G2 type scaffolds (Figure 3.14 - Figure 3.16).

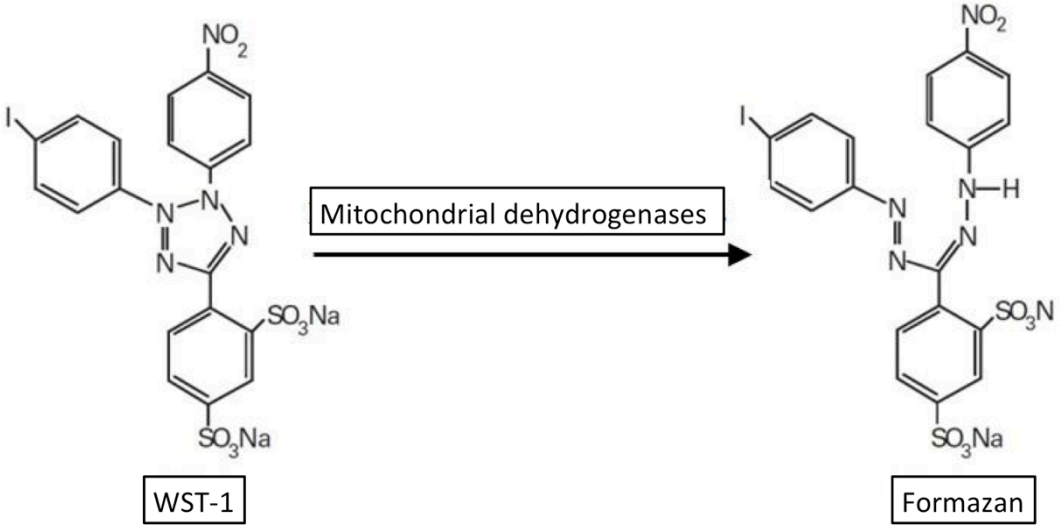


Figure 3.11 WST-1 to Formazan reaction by cellular enzymes [79]

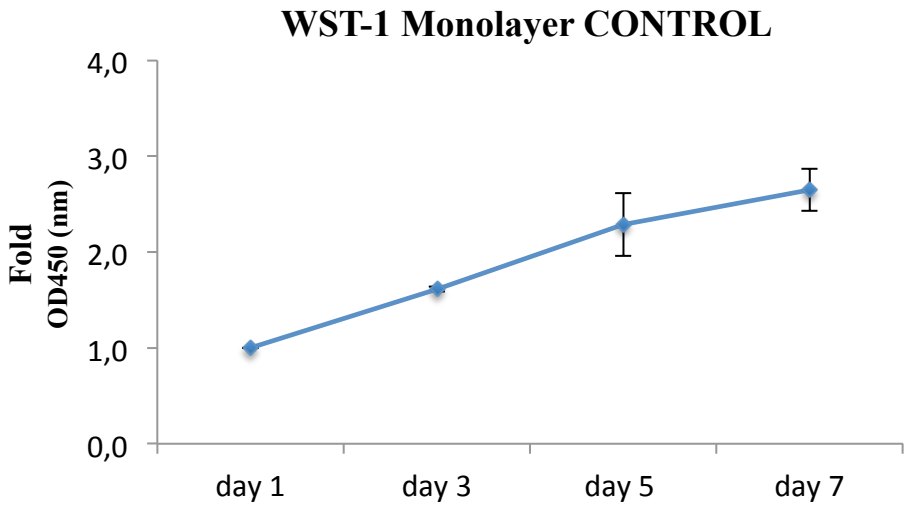


Figure 3.12 WST-1 assay**,** proliferation of control hMSC line seeded on tissue culture plates. Data graphed according the fold values of day 3,5,7 to day 1.

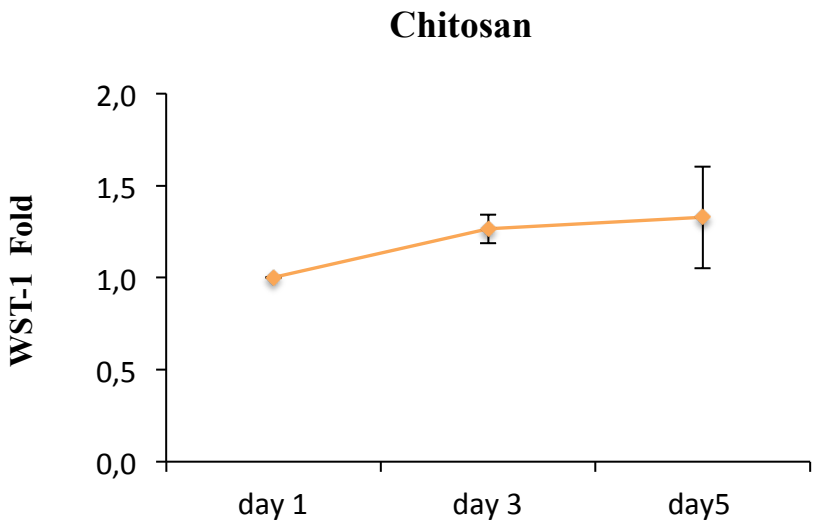


Figure 3.13 Proliferation according to WST-1 assay for cells cultured on only chitosan scaffolds. Data graphed according the fold values of day 3 and day 5 to day 1.

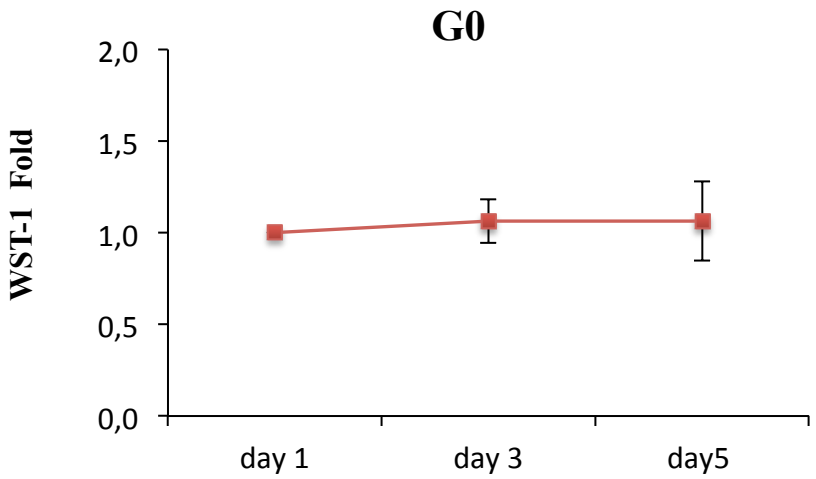


Figure 3.14 Graph showing the rate of proliferation according to WST-1 assay, for cells cultured on G0 PAMAM dendrimer incorporated chitosan scaffolds. Data graphed according the fold values of day 3 and day 5 to day 1.

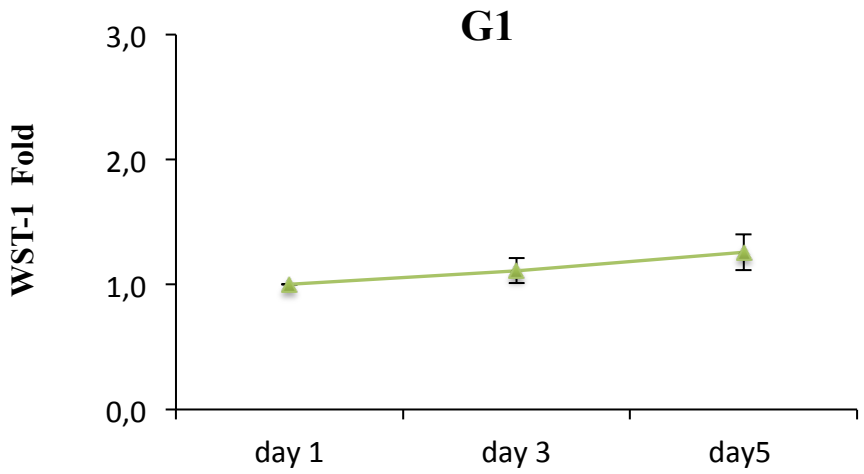


Figure 3.15 Graph showing the rate of proliferation according to WST-1 assay, for cells cultured on G1 PAMAM dendrimer incorporated chitosan scaffolds. Data graphed according the fold values of day 3 and day 5 to day 1.

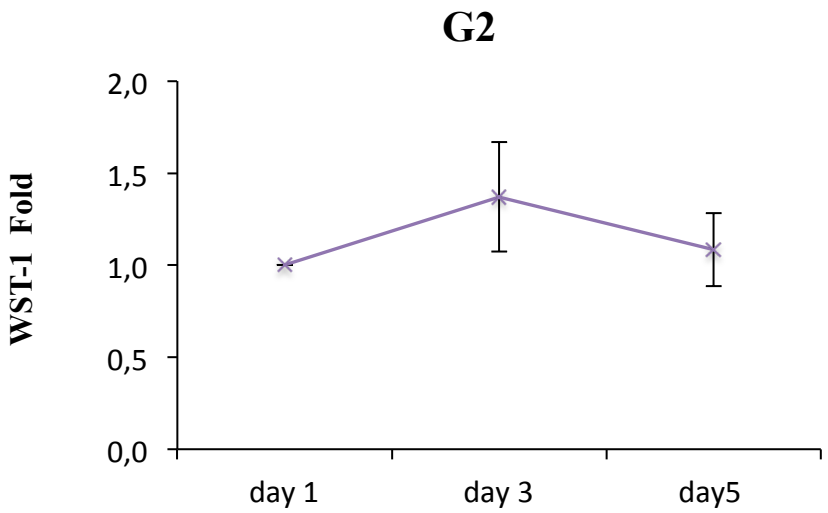


Figure 3.16 Graph showing the rate of proliferation according to WST-1 assay, for cells cultured on G2 PAMAM dendrimer incorporated chitosan scaffolds. Data graphed according the fold values of day 3 and day 5 to day 1.

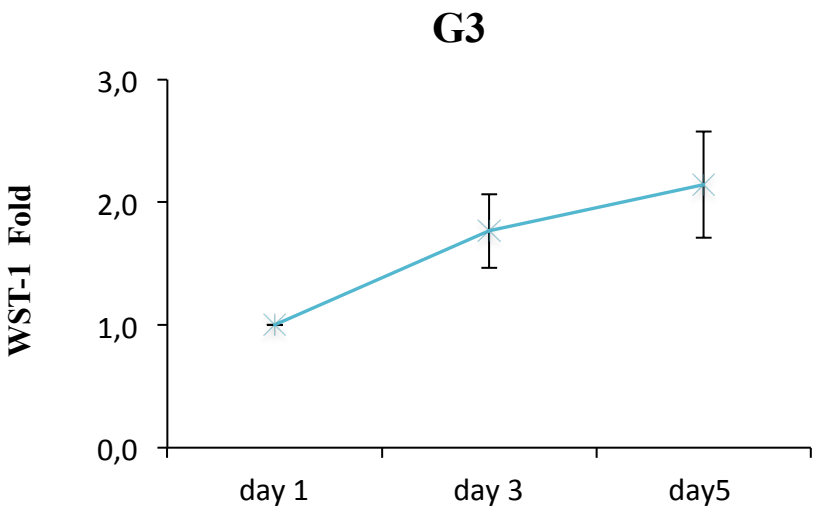


Figure 3.17 Graph showing the rate of proliferation according to WST-1 assay, for cells cultured on G3 PAMAM dendrimer incorporated chitosan scaffolds. Data graphed according the fold values of day 3 and day 5 to day 1.

For hMSCs seeded on different types of scaffold percentage of proliferation was calculated. The proliferation rate of hMSCs on only chitosan scaffold was chosen as control value so the absorbance values of PAMAM incorporated scaffolds were normalized to CTS values for the each particular time point of data. CTS scaffolds were assumed as 100% proliferative and PAMAM incorporated scaffolds were analyzed accordingly. G1 scaffold graphed under the CTS line with 85-90% of proliferation while others showed more than 100% values (Figure 3.18). G3 showed significant proliferation when compared to CTS control proliferation value by showing of a maximum 170% proliferation on day 5 (Figure 3.18).

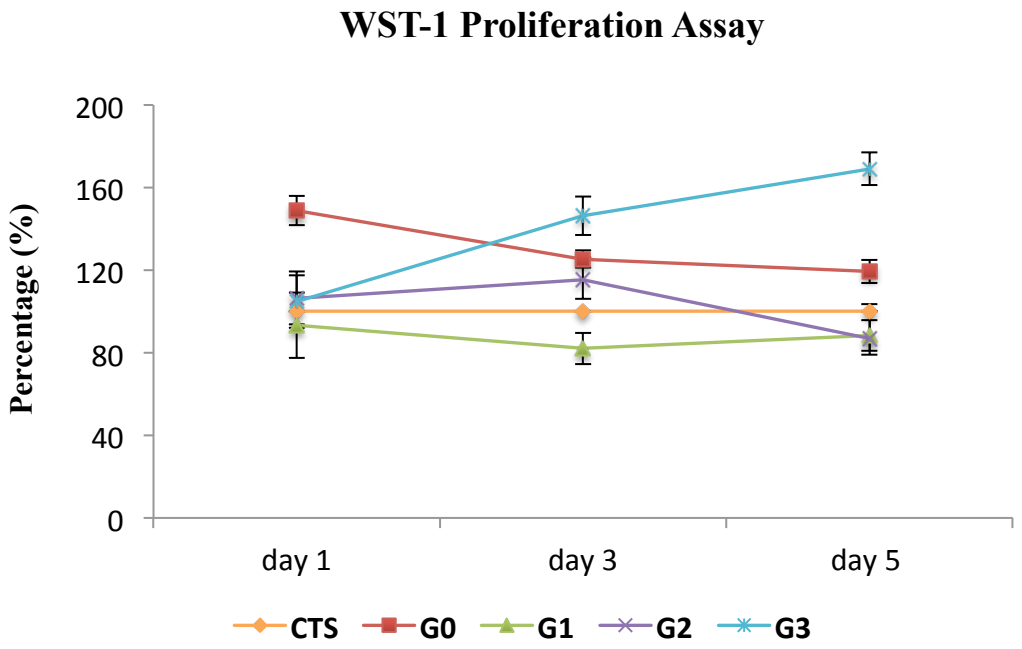


Figure 3.18 Graph showing the difference in percentage of proliferation for hMSC line seeded on different types of scaffolds. Data normalized to value of only chitosan scaffold.

3.7.2 LDH Cytotoxicity Assay

Lactate dehydrogenase (LDH) is an enzyme that is produced in mitochondria of all cells. When cells are dead, they secrete all its enzymes to its microenvironment. If the substrate of LDH is present the enzymatic reaction occurs and this can be measured by spectrophotometric techniques. Like WST-1 the medium changes its color to a deeper red color when the substrate is added into cultured cells (Figure 3.19).

The result of LDH assay showed very little cytotoxicity for monolayer control of hMSCs seeded on tissue culture plates. At the end of seven days of culture cumulative cytotoxicity percentage was maximum 15% (Figure 3.20). When hMSC line seeded scaffold were analyzed cumulatively, all types showed less than 30% cytotoxicity. And when analyzed separately, CTS scaffold showed maximum 14% of cytotoxicity whereas G3 scaffold showed least cytotoxic effect by maximum 9% cytotoxicity at the end of one week (Figure 3.21).

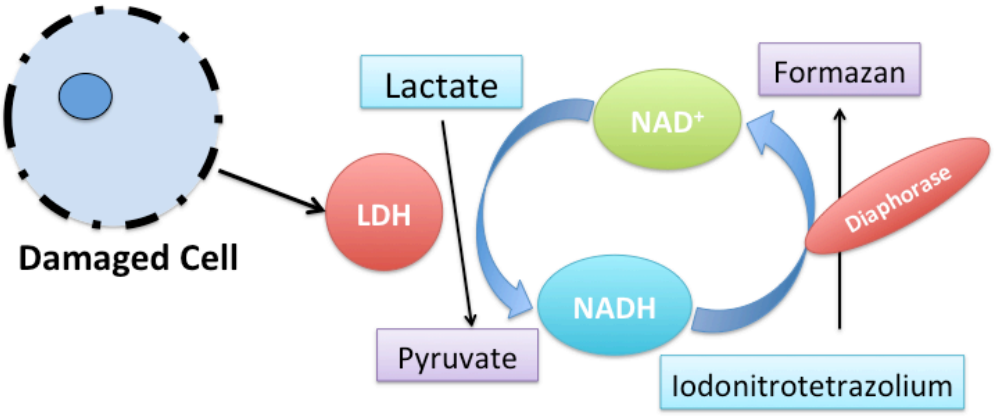


Figure 3.19 LDH mechanism.

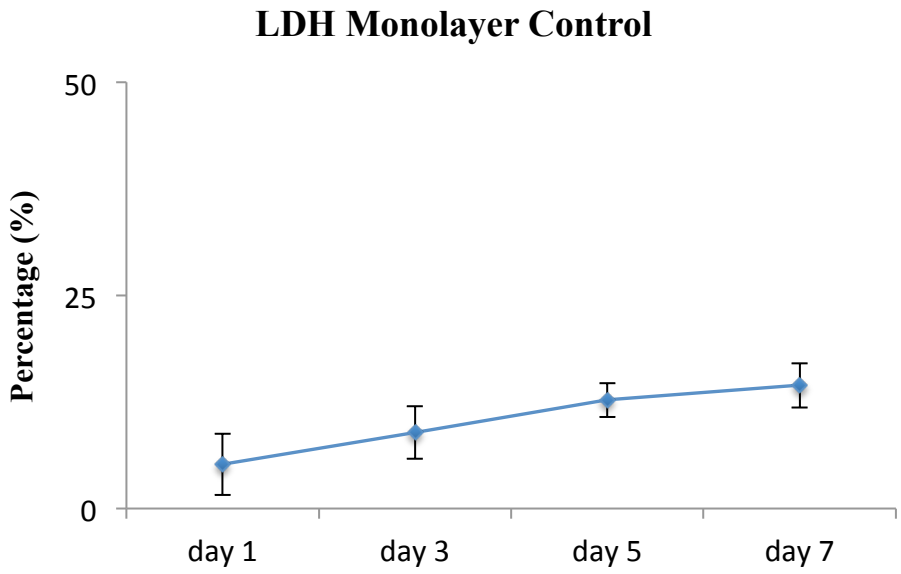


Figure 3.20 Cumulative percentage toxicity of hMSCs seeded on cell culture plates, data normalized to simultaneous WST-1 proliferation ratios in respective days.

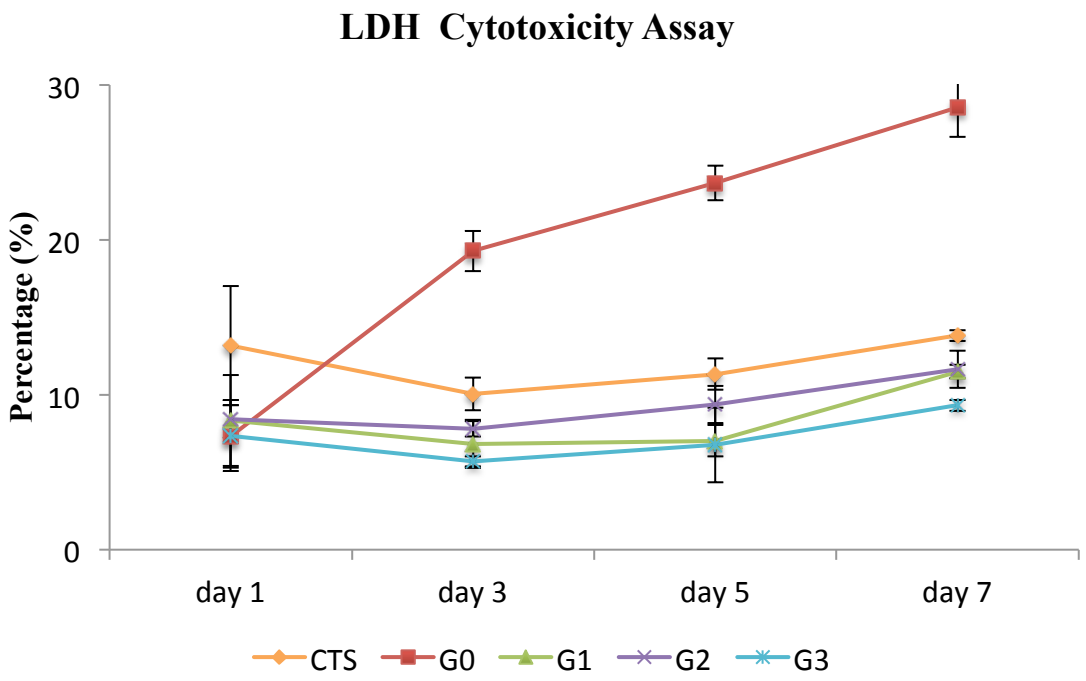
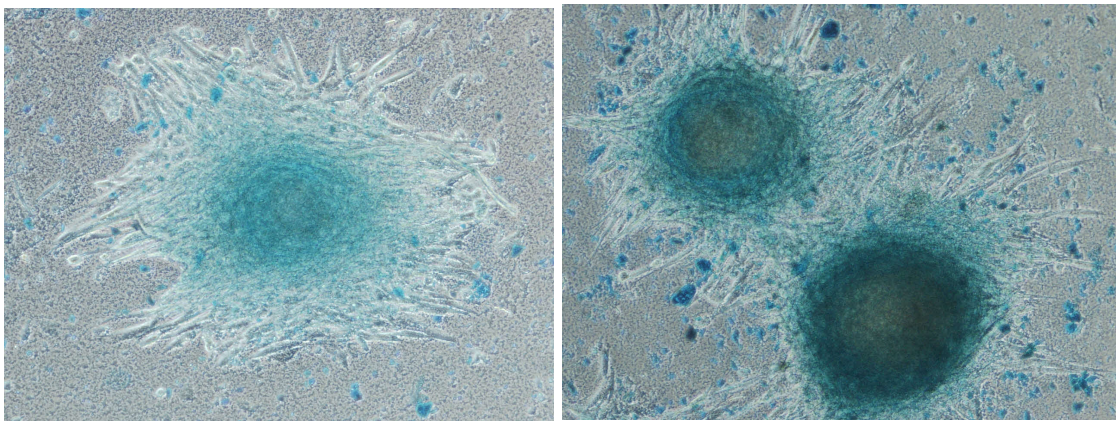


Figure 3.21 Cumulative percentage toxicity of hMSCs on scaffolds, data normalized to simultaneous WST-1 proliferation ratios in respective days.

3.8 CHONDROGENIC DIFFERENTIATION OF hMSC LINE CELLS

3.8.1 Chondrogenesis and Alcian Blue Staining



3.22 Differentiated hMSC line into chondrogenic cells.

hMSC line was differentiated into chondrogenic cells with StemPro kit. The assessment of chondrogenesis for differentiated cells was done with Alcian Bue Staining protocol. hMSC line was treated with chondrogenic medium and chondrogenic supplement for three weeks. The Alcian Blue Staining at the end of three weeks showed that hMSC line could be differentiated into chondrogenic cells (Figure 3.22). Staining was positive under light microscope.

CHAPTER 4

DISCUSSIONS AND CONCLUSIONS

In the last 15 years, tissue engineering has taken great developments in science. Starting from 1900s, with the developments in transplantation of an organ, transplantation of cells and developments in cell biology and material science make the understanding of tissue engineering today [3-5,8,10]. Tissue engineering aims the regeneration of damaged or lost tissue and organ with the help of cells and certain materials starting with *in vitro* studies [26, 80-81]. The main requirements of tissue engineering are; a suitable cell source that can be isolated from the body and suitable to be expanded *in vitro*, a scaffold that is produced from a biocompatible and biodegradable product, attachable by cells and porous in structure for biomolecules to reach the cells, and these two component should be provided by the third component which are the biological microenvironment that can help the cells to mimic its original tissue environment [6-9]. This biological microenvironment mainly constituted by required nutrients of cells and growth factors [7-8].

In the design of suitable scaffold, it is important to use materials similar to extracellular matrix of cells. This enables cells to be adjusted to this *in vitro* environment better and to be attached by these cells in order to allow their proliferation and expansion on the scaffold. Scaffolds can be prepared by either protein-based materials or carbohydrate-based materials [26,80-84], which can be synthetic [17,25-26,85] or natural polymers in nature [18-24]. Synthetic polymers are preferred with their ease of production in laboratory and being acceptable by living tissues in general. Natural polymers are preferred because of their similarity in structure with the components in the ECM, which make them better candidates for tissue engineering studies. In this respect one of the natural materials, chitosan is in great interest for

scientists in this field [29]. Chitosan is the deacetylated version of chitin that is one of the most abundant natural product in nature. The best feature of chitosan is that it enables porous structure with great versatility [29-31]. Its cationic nature and variable character to different pH makes it great for modification to design a scaffold of desired [29]. Since it is similar to GAGs in ECM [31], it makes a great base for tissues and this base can be incorporated for further ease of attachment.

With the development in cell biology discovery of ES cells and further ASCs has a great role in tissue engineering. Stem cells are cells that have self-renewal and proliferation capacity with a great potency to differentiate into any cell in the body [60-63]. They are classified according to their potency to differentiate into other cell lineages [65]. ES cells are considered to be pluripotent and can differentiate into almost any cell type in the body whereas ASCs are considered to be multipotent and can differentiate into certain cell types [65]. Since ES cell usage in research has ethical problems ASCs are more preferred [86]. MSCs are one type of ASC that can differentiate into cells with mesenchyme origin and also reported to transdifferentiate into non-mesenchymal origin also [62-64]. MSCs can be isolated from umbilical cord blood, dental pulp, adipose tissue and bone marrow [66]. They make a great choice of cell for tissue engineering because of their versatility to differentiate, ability to be expandable in vitro and their immunosuppressive effects on tissues [67-69].

In this study the chondrogenic properties of chondrogenic differentiation induced hMSCs seeded on PAMAM incorporated chitosan scaffolds was investigated. PAMAM incorporated chitosan scaffolds were prepared with freeze-drying method [87]. Characterization of scaffolds were experimented with swelling, water uptake and porosity measurement. The cell source of choice was immortalized bone marrow derived human mesenchymal stem cells. The indefinite proliferation capacity and obtaining large quantities of cell number for cell seeding makes them the most suitable cell type for this study.

In this study amine groups are added onto chitosan with different generations of modification. Amine groups created more surface area for cells to attach and this protein-structured environment can mimic the ECM for cells [88-89]. Amine groups modification to chitosan is a novel study for tissue engineering purposes, although earlier studied made amine group modification to chitosan, most of them were either a

chemical field study or as a carrier molecule for drugs or chemicals [54-59]. We tried to produce a scaffold that cells can attach with the addition of dendrimer like amine groups to chitosan aiming to study cell attachment, cultivation and tissue engineering.

In this study we used bone marrow-derived hMSC line [71-72]. We showed that hMSC line is suitable for *in vitro* study of tissue engineering. They have faster proliferation when compared to primer culture of hMSCs so they are suitable when high concentration of cell number is required. In the study of Shimomura T. et al., hepatic differentiation of this specific cell line was performed successfully [73]. In monolayer culture we successfully performed chondrogenic differentiation capability of these cells in vitro and confirmed with Alcian Blue staining (Figure 3.22).

All types of scaffolds (only chitosan and PAMAM incorporated scaffolds) were examined with SEM. The SEM images show that all the scaffold types are porous in structure as desired. The images show that non-incorporated chitosan has larger pores among all the types of scaffolds. With the addition of generations of amine groups, pore sizes get smaller slightly with each increasing generation. The difference in the appearance of the pore sizes is the best observed between non-incorporated chitosan and G3 chitosan (Figure 3.13 and Figure 3.17). The slight decrease in pore size does not affect the cell attachment since even G3 incorporated scaffold have pore size enough to let cells to migrate and attach inside the scaffold. Also the SEM images with higher magnification showed that all the scaffolds have a very smooth surface in micro scale.

In terms of porosity, swelling and water uptake all types of scaffolds show similar characteristics. They showed that they can take water up to 25% of their initial weight and they can swell up to 30% of their initial size. Also they are all almost 95% porous in structure.

We performed lysozyme degradation assay to see if natural enzymes that can be found in the body, can degrade the chitosan-based scaffolds. We examined the weight loss of the scaffolds in four weeks. CTS showed least degradation that is up to 10%. And all the other types of scaffold with PAMAM incorporation showed more than CTS scaffold but overall degradation did not pass 30%. The G3 scaffold with most PAMAM incorporation degradation value fall in average in all types and showed a 20% maximum degradation at the end of four weeks. Degradation of scaffolds is especially

important for future studies that would feature transplantation of these scaffolds to body. And the behavior of the scaffolds may differ *in vivo* and also the tissue environment where it is transplanted or the size of the transplantation. For better understanding of biodegradation process, *in vivo* and different variable studies should be performed.

Cell attachment onto scaffolds were confirmed with DAPI staining [90]. We observed nuclei inside the scaffold with fluorescence microscope. The initial attachment assessment was determined simply by counting the non-attached cells four hours after seeding cells onto scaffolds. The non-incorporated chitosan showed slightly better initial attachment when compared to scaffolds with PAMAM modification but all the scaffold types showed 75% of initial attachment (Figure 3. 9).

We have done cytotoxicity and cell proliferation assays in order to understand if the scaffolds that we designed have any toxic effect on cells. The monolayer control was done by seeding of hMSCs onto tissue culture plates that showed significant increase in proliferation in one-week time. The proliferation rate was evaluated by calculating the rate of increase comparing the other day values to first day value. In this respect, the relative ratio to first day absorbance value proliferation was graphed. CTS and G3 scaffolds showed more significant fold values than the other types of scaffolds. And G3 had most significant fold value compared the others. We also calculated the percentage of proliferation compared to CTS as control value. Since the behavior of cells would differ between monolayer culture conditions and scaffold conditions. Monolayer culture provides a two-dimensional environment while scaffolds provide a three-dimensional environment to cells. The CTS values were regarded as absolute and percentage of proliferation for PAMAM incorporated scaffolds were calculated for respective days. G3 incorporated scaffolds showed highest proliferation rate when compared to other types of generations and especially to non-incorporated chitosan scaffold. This can be due to number of amine groups that G3 scaffolds have that would mimic the ECM of cells more than non-incorporated chitosan.

Cytotoxicity results were also coherent with WST-1 assay. Very low cytotoxicity was observed in the LDH assay. In this respect, when we compared the non-incorporated scaffold and the chitosan with highest modification (G3), we saw significant difference. The proliferative result of G3 is better than non-incorporated chitosan and also in terms of cytotoxicity, the cumulative percentage for cytotoxicity

shoed that G3 with a value of 9% is less toxic than non-incorporated chitosan with a value of 13% (Figure 3.21). By taking the proliferation and cytotoxicity results into consideration, we can say that although initial attachment seems better in non-incorporated chitosan, in long-term cultivation of cells, G3 scaffold with highest PAMAM incorporation is better.

In conclusion, we showed that hMSC line is suitable for tissue engineering studies that could also feature differentiation of cells in future studies. Dendrimer incorporation to chitosan for tissue engineering purposes is studied for the first time, which makes this study novel in this aspect. The highest modification of amine groups onto chitosan showed better results in terms of proliferation rate and cytotoxicity when compared to non-incorporated chitosan scaffold. For the future study, biodegradation of chitosan scaffolds *in vivo* should be better understood and also differentiation of cells to desired tissue type on chitosan-based scaffolds should be performed with confirmative RNA analysis. And then cell-seeded scaffolds with the desired differentiation should be studied for *in vivo* transplantation to show practical aspect of this tissue engineering study.

REFERENCES

[1] Atala, A., "Engineering organs", *Curr Opin Biotechnol*, Vol. 20(5), pp. 575-592, 2009.

[2] Yoo, J. J., et al., "Regenerative medicine strategies for treating neurogenic bladder", *Int Neurourol J*, Vol. 15(3), pp. 109-119, 2011.

[3] Carrel, A. and Lindbergh, C. A., "The Culture of Whole Organs", *Science*, Vol. 81(2112), pp. 621-623, 1935.

[4] Guild, W. R., et al., "Successful homotransplantation of the kidney in an identical twin", *Trans Am Clin Climatol Assoc*, Vol. 67, pp. 167-173, 1955.

[5] Thomson, J. A., et al., "Embryonic stem cell lines derived from human blastocysts", *Science*, Vol. 282(5391), pp. 1145-1147, 1998.

[6] Hasetine, W., "A brave new medicine. A conversation with William Haseltine.. Interview by Joe Flower", *Health Forum J*, Vol. 42(4), pp. 28-30, 61-25, 1999.

[7] George, J. H. S., "Engineering of Fibrous Scaffolds for Use in Regenerative Medicine", in *Department of Materials*, Imperial Collage of London, 2009.

[8] Langer, R. and Vacanti, J. P., "Tissue engineering", *Science*, Vol. 260(5110), pp. 920-926, 1993.

[9] Sundelacruz, S. and Kaplan, D. L., "Stem cell- and scaffold-based tissue engineering approaches to osteochondral regenerative medicine", *Semin Cell Dev Biol*, Vol. 20(6), pp. 646-655, 2009.

[10] Cao, Y., et al., "Transplantation of chondrocytes utilizing a polymer-cell construct to produce tissue-engineered cartilage in the shape of a human ear", *Plast Reconstr Surg*, Vol. 100(2), pp. 297-302; discussion 303-294, 1997.

[11] Schmidt, D., et al., "Prenatally fabricated autologous human living heart valves based on amniotic fluid derived progenitor cells as single cell source", *Circulation*, Vol. 116(11 Suppl), pp. I64-70, 2007.

[12] Ramaswamy, S., et al., "The role of organ level conditioning on the promotion of engineered heart valve tissue development in-vitro using mesenchymal stem cells", *Biomaterials*, Vol. 31(6), pp. 1114-1125, 2010.

[13] Macchiarini, P., et al., "Clinical transplantation of a tissue-engineered airway", *Lancet*, Vol. 372(9655), pp. 2023-2030, 2008.

[14] Kuroyanagi, Y., et al., "Tissue-engineered product: allogeneic cultured dermal substitute composed of spongy collagen with fibroblasts", *Artif Organs*, Vol. 25(3), pp. 180-186, 2001.

[15] Steward, A. J., Liu Y., Wagner D.R., "Engineering Cell Attachments to Scaffolds in Cartilage Tissue Engineering", *JOM*, Vol. 63(4), 2011.

[16] Chao, P. H., Grayson, W., and Vunjak-Novakovic, G., "Engineering cartilage and bone using human mesenchymal stem cells", *J Orthop Sci*, Vol. 12(4), pp. 398-404, 2007.

[17] Frenkel, S. R. and Di Cesare, P. E., "Scaffolds for articular cartilage repair", *Ann Biomed Eng*, Vol. 32(1), pp. 26-34, 2004.

[18] Glowacki, J. and Mizuno, S., "Collagen scaffolds for tissue engineering", *Biopolymers*, Vol. 89(5), pp. 338-344, 2008.

[19] Bensaid, W., et al., "A biodegradable fibrin scaffold for mesenchymal stem cell transplantation", *Biomaterials*, Vol. 24(14), pp. 2497-2502, 2003.

[20] Wang, C. C., et al., "A highly organized three-dimensional alginate scaffold for cartilage tissue engineering prepared by microfluidic technology", *Biomaterials*, Vol. 32(29), pp. 7118-7126, 2011.

[21] Loken, S., et al., "Bone marrow mesenchymal stem cells in a hyaluronan scaffold for treatment of an osteochondral defect in a rabbit model", *Knee Surg Sports Traumatol Arthrosc*, Vol. 16(10), pp. 896-903, 2008.

[22] Wang, Y., et al., "Cartilage tissue engineering with silk scaffolds and human articular chondrocytes", *Biomaterials*, Vol. 27(25), pp. 4434-4442, 2006.

[23] Madihally, S. V. and Matthew, H. W., "Porous chitosan scaffolds for tissue engineering", *Biomaterials*, Vol. 20(12), pp. 1133-1142, 1999.

[24] Ko, Y. G., et al., "Preparation of chitosan scaffolds with a hierarchical porous structure", *J Biomed Mater Res B Appl Biomater*, Vol. 93(2), pp. 341-350, 2010.

[25] Tanaka, Y., et al., "Evaluation of the implant type tissue-engineered cartilage by scanning acoustic microscopy", *J Biosci Bioeng*, Vol. 113(2), pp. 252-257, 2012.

[26] Kim, B.-S., et al., "Design of artificial extracellular matrices for tissue engineering", *Progress in Polymer Science*, Vol. 36(2), pp. 238-268, 2011.

[27] Jayakumar, R., et al., "Biomaterials based on chitin and chitosan in wound dressing applications", *Biotechnol Adv*, Vol. 29(3), pp. 322-337, 2011.

[28] Alves da Silva, M. L., et al., "Chitosan/polyester-based scaffolds for cartilage tissue engineering: Assessment of extracellular matrix formation", *Acta Biomaterialia*, Vol. 6(3), pp. 1149-1157, 2010.

[29] Kim, I.-Y., et al., "Chitosan and its derivatives for tissue engineering applications", *Biotechnology Advances*, Vol. 26(1), pp. 1-21, 2008.

[30] Cardea, S., Pisanti, P., and Reverchon, E., "Generation of chitosan nanoporous structures for tissue engineering applications using a supercritical fluid assisted process", *The Journal of Supercritical Fluids*, Vol. 54(3), pp. 290-295, 2010.

[31] Suh, J. K. and Matthew, H. W., "Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review", *Biomaterials*, Vol. 21(24), pp. 2589-2598, 2000.

[32] Hirano, S., Tsuchida, H., and Nagao, N., "N-acetylation in chitosan and the rate of its enzymic hydrolysis", *Biomaterials*, Vol. 10(8), pp. 574-576, 1989.

[33] Onishi, H. and Machida, Y., "Biodegradation and distribution of water-soluble chitosan in mice", *Biomaterials*, Vol. 20(2), pp. 175-182, 1999.

[34] Elcin, Y. M., et al., "Xenotransplantation of fetal porcine hepatocytes in rats using a tissue engineering approach", *Artif Organs*, Vol. 23(2), pp. 146-152, 1999.

[35] Nishimura, K., et al., "Immunological activity of chitin and its derivatives", *Vaccine*, Vol. 2(1), pp. 93-99, 1984.

[36] Haleem, A. M. and Chu, C. R., "Advances in Tissue Engineering Techniques for Articular Cartilage Repair", *Operative Techniques in Orthopaedics*, Vol. 20(2), pp. 76-89, 2010.

[37] Kratz, G., et al., "Heparin-chitosan complexes stimulate wound healing in human skin", *Scand J Plast Reconstr Surg Hand Surg*, Vol. 31(2), pp. 119-123, 1997.

[38] Chen, G., et al., "The use of a novel PLGA fiber/collagen composite web as a scaffold for engineering of articular cartilage tissue with adjustable thickness", *Journal of Biomedical Materials Research Part A*, Vol. 67A(4), pp. 1170-1180, 2003.

[39] Ameer, G. A., Mahmood, T. A., and Langer, R., "A biodegradable composite scaffold for cell transplantation", *J Orthop Res*, Vol. 20(1), pp. 16-19, 2002.

[40] Wayne, J. S., et al., "In vivo response of polylactic acid-alginate scaffolds and bone marrow-derived cells for cartilage tissue engineering", *Tissue Eng*, Vol. 11(5-6), pp. 953-963, 2005.

[41] Schaefer, D., et al., "In vitro generation of osteochondral composites", *Biomaterials*, Vol. 21(24), pp. 2599-2606, 2000.

[42] Moutos, F. T. and Guilak, F., "Composite scaffolds for cartilage tissue engineering", *Biorheology*, Vol. 45(3-4), pp. 501-512, 2008.

[43] Cervantes, T. M., et al., "Design of composite scaffolds and three-dimensional shape analysis for tissue-engineered ear", *J R Soc Interface*, Vol. 10(87), pp. 20130413, 2013.

[44] Qi, B. W., et al., "Chitosan/poly(vinyl alcohol) hydrogel combined with Ad-hTGF-beta1 transfected mesenchymal stem cells to repair rabbit articular cartilage defects", *Exp Biol Med (Maywood)*, Vol. 238(1), pp. 23-30, 2013.

[45] Wright, L., et al., "PDLA/PLLA and PDLA/PCL nanofibers with a chitosan-based hydrogel in composite scaffolds for tissue engineered cartilage", *J Tissue Eng Regen Med*, 2012.

[46] Zhang, P., et al., "RGD-conjugated copolymer incorporated into composite of poly(lactide-co-glycotide) and poly(L-lactide)-grafted nanohydroxyapatite for bone tissue engineering", *Biomacromolecules*, Vol. 12(7), pp. 2667-2680, 2011.

[47] Versace, D. L., et al., "Versatile photochemical surface modification of biopolyester microfibrous scaffolds with photogenerated silver nanoparticles for antibacterial activity", *Adv Healthc Mater*, Vol. 2(7), pp. 1008-1018, 2013.

[48] Ragaseema, V. M., et al., "The antithrombotic and antimicrobial properties of PEG-protected silver nanoparticle coated surfaces", *Biomaterials*, Vol. 33(11), pp. 3083-3092, 2012.

[49] Bongio, M., et al., "Biomimetic modification of synthetic hydrogels by incorporation of adhesive peptides and calcium phosphate nanoparticles: in vitro evaluation of cell behavior", *Eur Cell Mater*, Vol. 22, pp. 359-376, 2011.

[50] Astruc, D., Boisselier, E., and Ornelas, C., "Dendrimers designed for functions: from physical, photophysical, and supramolecular properties to applications in sensing, catalysis, molecular electronics, photonics, and nanomedicine", *Chem Rev*, Vol. 110(4), pp. 1857-1959, 2010.

[51] Svenson, S. and Tomalia, D. A., "Dendrimers in biomedical applications--reflections on the field", *Adv Drug Deliv Rev*, Vol. 57(15), pp. 2106-2129, 2005.

[52] Sashiwa, H., Shigemasa, Y., and Roy, R., "Chemical modification of chitosan. Part 9: Reaction of N-carboxyethylchitosan methyl ester with diamines of acetal ending PAMAM dendrimers", *Carbohydr. Polym.*, Vol. 47(2), pp. 201, 2002.

[53] Sashiwa, H., Shigemasa, Y., and Roy, R., "Chemical modification of chitosan 11: chitosan–dendrimer hybrid as a tree like molecule", *Carbohydr. Polym.*, Vol. 49(2), pp. 195, 2002.

[54] Winnicka, K., et al., "The effect of PAMAM dendrimers on the antibacterial activity of antibiotics with different water solubility", *Molecules*, Vol. 18(7), pp. 8607-8617, 2013.

[55] Ozturk, K., et al., "Cytotoxicity and in vitro characterization studies of synthesized Jeffamine-cored PAMAM dendrimers", *J Microencapsul*, 2013.

[56] Liu, K. C. and Yeo, Y., "Zwitterionic chitosan-polyamidoamine dendrimer complex nanoparticles as a pH-sensitive drug carrier", *Mol Pharm*, Vol. 10(5), pp. 1695-1704, 2013.

[57] Hui Zhong, et al., "Studies on Polyamidoamine Dendrimers as Efficient Gene Delivery Vector", *Journal of Biomaterials Applications*, Vol. 22(6), pp. 527-544, 2008.

[58] Zhang, X. Q., et al., "In vitro gene delivery using polyamidoamine dendrimers with a trimesyl core", *Biomacromolecules*, Vol. 6(1), pp. 341-350, 2005.

[59] Posocco, P., et al., "Impact of siRNA Overhangs for Dendrimer-Mediated siRNA Delivery and Gene Silencing", *Mol Pharm*, Vol. 10(8), pp. 3262-3273, 2013.

[60] Weissman, I. L., Anderson, D. J., and Gage, F., "Stem and progenitor cells: Origins, phenotypes, lineage commitments, and transdifferentiations", *Annual Review of Cell and Developmental Biology*, Vol. 17, pp. 387-403, 2001.

[61] Lensch, M. W., Daheron, L., and Schlaeger, T., "Pluripotent stem cells and their niches", *Stem Cell Reviews*, Vol. 2(3), pp. 185-201, 2006.

[62] Passier, R. and Mummery, C., "Origin and use of embryonic and adult stem cells in differentiation and tissue repair", *Cardiovasc Res*, Vol. 58(2), pp. 324-335, 2003.

[63] Zaim, M., "Neural Differentiation Potential Variability of Bone Marrow Derived Human Mesenchymal Stem Cells", in *Department of Biology*, Fatih University, 2011.

[64] Young, H. E. and Black, A. C., Jr., "Adult stem cells", *Anat Rec A Discov Mol Cell Evol Biol*, Vol. 276(1), pp. 75-102, 2004.

[65] Fernández Vallone, V. B., et al., "Mesenchymal stem cells and their use in therapy: What has been achieved?", *Differentiation*, Vol. 85(1–2), pp. 1-10, 2013.

[66] Minguell, J. J., Erices, A., and Conget, P., "Mesenchymal stem cells", *Exp Biol Med (Maywood)*, Vol. 226(6), pp. 507-520, 2001.

[67] Ben-Ami, E., Berrih-Aknin, S., and Miller, A., "Mesenchymal stem cells as an immunomodulatory therapeutic strategy for autoimmune diseases", *Autoimmun Rev*, Vol. 10(7), pp. 410-415, 2011.

[68] Ringden, O. and Le Blanc, K., "Mesenchymal stem cells for treatment of acute and chronic graft-versus-host disease, tissue toxicity and hemorrhages", *Best Pract Res Clin Haematol*, Vol. 24(1), pp. 65-72, 2011.

[69] Caplan, A. I., "Adult mesenchymal stem cells for tissue engineering versus regenerative medicine", *J Cell Physiol*, Vol. 213(2), pp. 341-347, 2007.

[70] Pountos, I., et al., "Mesenchymal stem cell tissue engineering: techniques for isolation, expansion and application", *Injury*, Vol. 38 Suppl 4, pp. S23-33, 2007.

[71] Mori, T., et al., "Combination of hTERT and bmi-1, E6, or E7 induces prolongation of the life span of bone marrow stromal cells from an elderly donor without affecting their neurogenic potential", *Mol Cell Biol*, Vol. 25(12), pp. 5183-5195, 2005.

[72] Takeda, Y., et al., "Can the life span of human marrow stromal cells be prolonged by bmi-1, E6, E7, and/or telomerase without affecting cardiomyogenic differentiation?", *J Gene Med*, Vol. 6(8), pp. 833-845, 2004.

[73] Shimomura, T., et al., "Hepatic differentiation of human bone marrow-derived UE7T-13 cells: Effects of cytokines and CCN family gene expression", *Hepatol Res*, Vol. 37(12), pp. 1068-1079, 2007.

[74] Takahashi, K. and Yamanaka, S., "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors", *Cell*, Vol. 126(4), pp. 663-676, 2006.

[75] Duan, X., et al., "Application of induced pluripotent stem (iPS) cells in periodontal tissue regeneration", *Journal of Cellular Physiology*, Vol. 226(1), pp. 150-157, 2011.

[76] Wen, Y., et al., "Application of induced pluripotent stem cells in generation of a tissue-engineered tooth-like structure", *Tissue Eng Part A*, Vol. 18(15-16), pp. 1677-1685, 2012.

[77] Hongxu, L., et al., "Culture of bovine articular chondrocytes in funnel-like collagen-PLGA hybrid sponges", *Biomedical Materials*, Vol. 6(4), pp. 045011, 2011.

[78] Marieb, E. N., *Essentials of Human Anatomy and Physiology*, 9th edBenjamin Cummings, 2008.

[79] Ragetly, G. R., et al., "Cartilage tissue engineering on fibrous chitosan scaffolds produced by a replica molding technique", *Journal of Biomedical Materials Research Part A*, Vol. 93A(1), pp. 46-55, 2010.

[80] [Cited 2013, 30.05.2013] Available from: http://www.biovision.com/images/k304.jpg

[81] Ghasemi-Mobarakeh, L., et al., "Advances in electrospun nanofibers for bone and cartilage regeneration", *J Nanosci Nanotechnol*, Vol. 13(7), pp. 4656-4671, 2013.

[82] Guasti, L., et al., "Chondrogenic differentiation of adipose tissue-derived stem cells within nanocaged POSS-PCU scaffolds: a new tool for nanomedicine", *Nanomedicine*, 2013.

[83] Tigli, R. S., et al., "Chondrogenesis in perfusion bioreactors using porous silk scaffolds and hESC-derived MSCs", *J Biomed Mater Res A*, Vol. 96(1), pp. 21-28, 2011.

[84] Altman, A. M., et al., "Adhesion, migration and mechanics of human adipose-tissue-derived stem cells on silk fibroin–chitosan matrix", *Acta Biomaterialia*, Vol. 6(4), pp. 1388-1397, 2010.

[85] Olson, A., Graver, A., and Grande, D., "Scaffolds for Articular Cartilage Repair", Vol. 22(3), pp. 219-227, 2012.

[86] Kastenberg, Z. J. and Odorico, J. S., "Alternative sources of pluripotency: science, ethics, and stem cells", *Transplantation reviews (Orlando, Fla.)*, Vol. 22(3), pp. 215-222, 2008.

[87] Chun Ho, K., et al., "Protocol for Freeze-Drying Method", *A Manual for Biomaterials/Scaffold Fabrication Technology*, pp. 91-99, WORLD SCIENTIFIC, 2007.

[88] Atala, A., et al., (editors), *Principles of Regenerative Medicine*. 2nd edition ed. Elsevier Inc., 2011.

[89] Di Martino, A., Sittinger, M., and Risbud, M. V., "Chitosan: A versatile biopolymer for orthopaedic tissue-engineering", *Biomaterials*, Vol. 26(30), pp. 5983-5990, 2005.

[90] Bhat, S. and Kumar, A., "Cell proliferation on three-dimensional chitosan-agarose-gelatin cryogel scaffolds for tissue engineering applications", *J Biosci Bioeng*, Vol. 114(6), pp. 663-670, 2012.