IN VITRO STUDIES OF CARBOXYMETHYL CELLULOSE/GELATIN AND CALCIUM PHOSPHATE/CALCIUM SULFATE CEMENT BASED COMPOSITES FOR BONE TISSUE ENGINEERING

by

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ABSTRACT

IN VITRO STUDIES OF CARBOXYMETHYL CELLULOSE/GELATIN AND CALCIUM PHOSPHATE/CALCIUM SULFATE CEMENT BASED COMPOSITES FOR BONE TISSUE ENGINEERING

In this study, a calcium phosphate (CPCs) which composed of tetracalcium phosphate (TTCP) and dicalcium phosphate dihydrate (DCPD) and calcium sulfate dihydrate (CSD)-based cement was introduced into carboxymethyl cellulose (CMC)-gelatin (Gel) and citric acid (CA) hydrogel for bone tissue engineering. In here, 2 w/v% CMC was mixed with 20 wt% CA and 10 wt% Gel to obtain the liquid phase. After that, 76.65% of TTCP was mixed with 23.35% of DCPD. This mixture was blended with 20 % of CSD in the total powder phase. Combined liquid and powder phases were molded in a syringe and set at 50°C for 72h. Morphology of the composites were examined by using Scanning Electron Microscopy (SEM). Physical characteristics of the composites were investigated with swelling, degradation and pH studies after incubation in PBS at 37°C. Cell culture studies were performed with bone marrow-derived mesenchymal stem cell (BMDMSCs). Cell viability was measured with Alamar Blue assay. Finally, in vitro cell adhesion was observed again by using SEM. The results indicated the homogenous structure of P62.5 and P65 and micropores in all composites. According to swelling-degradation results, except for P70, all the composites had the same swelling-degradation trend. At the end of 72h, when powder ratio was increased, the swelling degree was decreased. The powder ratio and degradation were inversely proportional. pH study showed that, at the end of 72h it reached around 7 which is similar with the physiological value for all composites. Cellular viability was calculated and only significant decrease was observed for P65 between 1-3 and 14 days. Overall, composites were successfully produced and according to results they had a potential for bone tissue engineering in terms of their biocompatibility.

Keywords: CPCs, CMC, Calcium sulfate, Composite, Bone, Biocompatibility

ÖZET

KEMİK DOKU MÜHENDİSLİĞİ İÇİN KARBOKSİMETİL SELÜLOZ/JELATİN VE KALSİYUM FOSFAT/KALSİYUM SÜLFAT SERAMİK BAZLI KOMPOZİTLER*İ IN VITRO* ÇALIŞMALARI

Bu çalışmada, kemik doku mühendisliği için tetrakalsiyum fosfat (TTCP) ve dikalsiyum fosfat dihidrattan (DCPD) oluşan kalsiyum fosfat ve kalsiyum sülfat dihidrattan (CSD) oluşan seramik,karboksimetil selüloz (CMC), jelatin (Gel) ve sitrik asitten (CA) oluşan hidrojelle karıstırılmıştır. Burada, 2 w/v% CMC, 20 wt% CA ve 10 wt% Gel ile karıştırılarak sıvı fazı oluşturmuştur. Daha sonra, 76.65 % TTCP, 23.35% DCPD ile karıştırılmıştır. Bu karışım, totalde 20% CSD olacak şekilde bir araya getirilmiştir. Birleştirilen sıvı ve katı faz, bir şırınga içinde 50°C de 72 saat kalıplandırılmıştır. Kompozitlerin morfolojileri Taramalı Elektron Mikroskobu (SEM) ile incelenmiştir. Kompozitlerin fiziksel karakteristikleri PBS içinde 37°C'de inkübe edildikten sonra, şişme, çözünme ve pH çalışmaları ile araştırılmıştır. Hücre kültürü çalışmaları kemik iliğinden elde edilen mezenkimal kök hücreler ile (BMDMSC) yapılmıştır. Hücre canlılığı Alamar Mavisi testi ile hesaplanmıştır. Son olarak hücre yapışması tekrar SEM kullanılarak gözlenmiştir. Sonuçlar P62.5 ve P65 kompozitlerinin homojen yapısını ve tüm kompozitlerin mikroporlu yapısını göstermiştir. Şişme-çözünme testine göre P70 hariç bütün kompozitler aynı şişme-çözünme eğilimini göstermiştir. 72 saat sonunda toz oranı arttıkça şişme oranı düşmüştür. Toz oranı ve çözünme ise ters orantılıdır. pH çalışmasına göre tüm kompozitler için 72 saat sonunda pH fizyolojik değere yakın olan 7'ye ulaşmıştır. Hücre canlılığı hesaplanmış ve yalnızca P65 için 1-3 ve 14. günlerde önemli bir düşüş görülmüştür. Totalde, kompozitler başarıyla üretilmiştir ve sonuçlara göre kemik doku mühendisliği için biyouyumluluk yönünden potansiyelleri vardır.

Anahtar Sözcükler: CPCs, CMC, Kalsiyum sülfat, Kompozit, Kemik, Biyouyumluluk

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

P62.5	Composite with 62.5% powder ratio
P65	Composite with 65% powder ratio
P67.5	Composite with 67.5% powder ratio
P70	Composite with 70% powder ratio
Ca^{2+}	Calcium ion
PO_4^{3-}	Phosphate ion
H^+	Hydrogen ion
OH^-	Hydroxide ion
H_3O^+	Hydronium ion
CaO^-	Calcium oxide
CO_2	Carbon dioxide
W_i	Initial weight
W_f	Final weight

LIST OF ABBREVIATIONS

ALP	Alkaline Phosphatase
BMDMSCs	Bone Marrow Derived Mesenchymal Stem Cells
CA	Citric Acid
CMC	Carboxymethyl Cellulose
CPC	Calcium Phosphate Cement
CSD	Calcium sulfate
DS	Degree of Substitution
DCPA	Di-calcium Phosphate Anhydrous
DCPD	Di-calcium Phosphate Di-hydrate
Gel	Gelatin
HA	Hydroxyapatite
SEM	Scanning Electron Microscopy
TCP	Tissue Culture Plate
TTCP	Tetra-calcium Phosphate
PBS	Phosphate Buffer Saline

1. INTRODUCTION

1.1 Motivation

Bone fractures are very common in society and its healing can be problematic. To overcome related problems, many different materials have been developed in medicine [1].However, each material has both advantages and disadvantages. For example, although steel and titanium implants have been used for a long time, they have corrosion risk which is toxic for the cellular environment and they also produce stress for surrounding bone tissue[2]. Ceramics such as zirconia or hydroxyapatite (HA) are also preferable in this area due to resemblance with natural bone. HA is the most preferable ceramic in studies because of its resemblance with the mineral phase of the bone but it has some drawbacks such as low solubility, unstable structure, and difficulties in shaping. Therefore, calcium phosphate cements (CPCs) are developed in the 1980s, which comprise of tetracalcium phosphate (TTCP) and dicalcium hydrogen phosphate (DCPD) or calcium sulfates such as calcium sulfate dehydrate (CSD) to overcome problems of HA. Also, HA which is deposited from CPCs in an aqueous environment such as PBS mimics the natural HA more than commercial CPCs. However, they are brittle which gives the disadvantage of weak support for newly formed tissue and also they have less surface area for suitable cellular attachment [2, 3]. Polymers are other beneficial options due to their high biocompatibility. Also, they can have tunable characteristics when they are cross-linked with different additives in their hydrogel form. By changing temperature, time or concentration of crosslinking agent and reaction, their swelling and degradation characteristics can be adjusted according to the application. However, their mechanical properties are low, solely polymers or hydrogels are not supportive enough for newly formed bone tissue [4].

Carboxymethyl cellulose (CMC) is a cellulose ester derivative which can be cross-linked with different chemicals such as glutaraldehyde. However, generally, these chemicals are toxic. Therefore, citric acid emerged as a non-toxic, economical solution

for crosslinking of CMC to produce hydrogels [5, 6]. Another polymer that can be integrated into this hydrogel form is gelatin [7, 8]. Gelatin in CPCs increase the cellular attachment, mechanical properties and control the porosity [9, 10]. Hence, gelatin addition has positive effects on both biocompatibility and physical features of the hydrogel.

Combination of different materials in tissue engineering is a developing perspective to overcome the disadvantages of combined materials as composites. There are many studies in the literature which had a combination of different polymers with directly HA or CPCs such as chitosan, collagen or PLGA [11, 12, 13, 14]. This combination can control the porosity, setting time, mechanical properties, degradation time, cellular integration capability of composites. However, degradation of CPCs can be toxic for the cells. Hence, CPCs integration with these polymers increase the cohesion of CPCs and an inflammatory response can be prevented. There is a dissolution-precipitation mechanism between polymer and CPCs. Ca^{2+} and PO_4^{3-} ions are diffused into the environment. Then, they can precipitate as HA on the deprotonated polymer [15]. All these studies gave a promising solution for bone tissue engineering, however, there are some studies state that collagen or chitosan can decrease compressive strength and have negative effects on degradation and plasticity of the composites [16, 17, 18, 19]. Thereby, using hydrogels in composites can be more advantageous to adjust the properties of material via mentioned tunability of crosslinking reaction.

From this point of view, producing hydrogel-CPCs based composites has the potential for bone tissue engineering. For this purpose, this work focused to produce CA cross-linked CMC-Gel hydrogel and mix it with TTCP-DCPD-CSD based CPC. These composites were investigated for physical and biological properties as swelling, degradation characteristics, pH changes, cell viability, and adhesion.

1.2 Objectives

The aim of the study is to produce a biocompatible composite material which is composed of CMC-Gel hydrogel and TTCP-DCPD-CSD powder phase to resolve complications of bone fractures.

- To produce CA cross-linked CMC-Gel hydrogel.
- To investigate the effect of the addition of TTCP-DCPD-CSD powder phase in CMC-Gel hydrogel with different powder to liquid ratio.
- To control the swelling-degradation features of the composite.
- To observe pH changes in the biological environment due to composite addition.
- To measure the viability of the Bone Marrow Derived Stem Cells (BMDMSCs) which cultured on composites.
- To examine the adhesion of the BMDMSCs on composite materials.

1.3 Outline

This thesis is composed of 5 chapters. Chapter 1 contains the motivation of the study and objectives. Structure and biology of the bone, potential bone tissue engineering solutions, and materials which are used in this study are introduced in the background section as Chapter 2. Chapter 3 states the materials and methods. Results of experiments clarify in chapter 4. Finally, in chapter 5 discussions of results and conclusions are presented.

2. BACKGROUND

2.1 Bone Structure

Bone tissue is composed of both organic and inorganic elements which is shown in Figure 2.1; therefore, it is called a multiphase structure. The organic phase of the bone has mainly flexible, deformable and tough type I collagen and non-collagenous proteins (NCP) such as glycoproteins like alkaline phosphatase (ALP), osteocalcin, osteonectin, osteopontin, and morphogenetic proteins. They constitute nearly 25 % of the total volume of the bone [20, 2, 21], a triple helical structure is shaped by the wrapping of left-handed helices around each other into a right-handed helix. In collagen, I triple helix structure composed of two $\alpha 1$ and one $\alpha 2$ chain. Glycine is the repeated aminoacid in the primary structure of this triple helix domain. Generally, alanine or proline and hydroxyproline chase the glycine in this structure. Here hydroxyproline is important for the stability of triple helix structure of collagen due to its capacity to bound water molecules via H bonding with its hydroxyl groups [21, 2]. Inorganic matrix of bone is formed by hydroxyapatite crystals (HA,$Ca_{10}(PO_4)_6(OH)_2$), citrate, sodium, magnesium, fluoride and calcium phosphatases. This mineral phase which is nearly 65 % of bone is the source of brittle and stiff structure. Apatite and other minerals are embedded into hole zones of collagen fibrils and pores which are between the fibrils. However, highly mineralized bone tissue become more brittle because mechanical integrity of the bone is demolished therefore, the risk of fracture is increased. The remaining part which is 10 % of the bone volume is water. Nevertheless, the role of water is not completely understood, it is assumed that water has a role in toughness of bone by acting as plasticizer[20, 22, 21].

Besides, as seen in Figure 2.2, there are two subtypes of bone tissue at the macro level as a cortical(compact) and trabecular(cancellous) bone. These tissues are classed according to porous structure, locations and functions [23, 21]. The cortical bone which occupies 80% of bone is very dense and have fewer blood vessels. The porosity

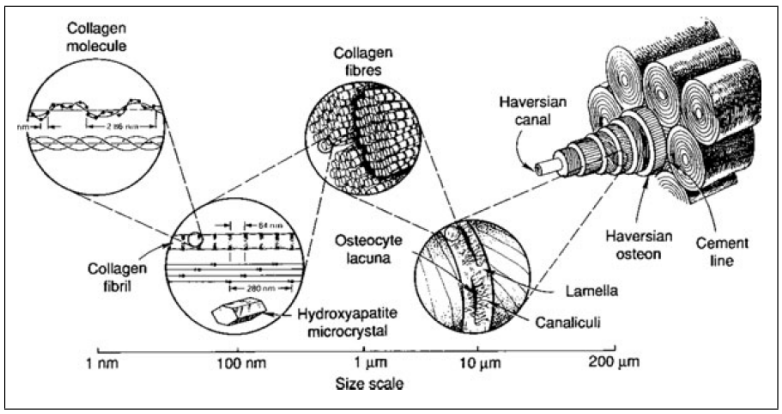

Figure 2.1 Structure of the bone [23].

of cortical bone is very low which is 3-5% with Haversian canals. Cancellous bone is surrounded by cortical bone tissue by this way tissue is supported and protected. Cancellous bone tissue that is filled an inside space of bone and edges of the long bones has a higher surface area and less dense structure. Trabeculae which are composed of mineral and collagen plates and cavities are main structures of cancellous bone tissue. Red bone marrow is also found in these cavities. These two different phases of bone provide various mechanical characteristics of bone [21, 20].

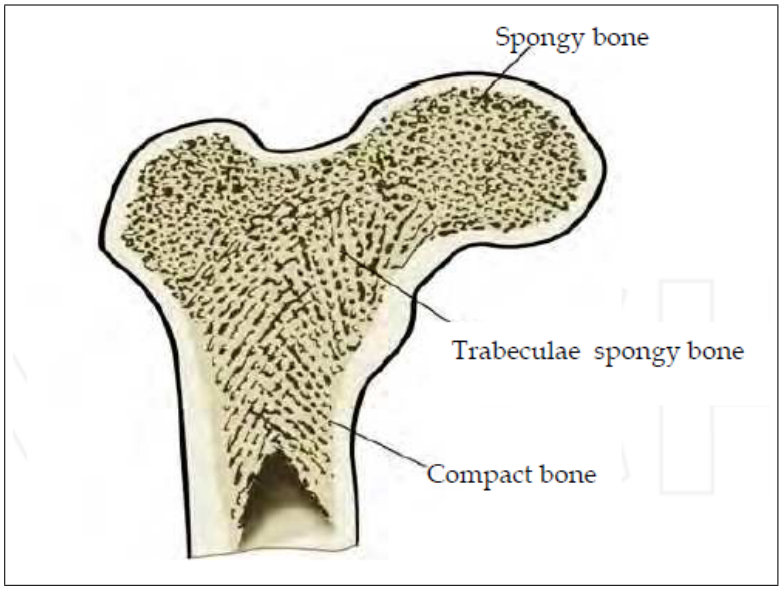

Figure 2.2 Subtypes of bone tissue [24].

2.2 Bone Biology

There are different types of cells which are derived from mesenchymal cells in bone tissue as osteoblasts, osteoclasts, and osteocytes. Osteoblasts are assigned to bone formation. They secrete the bone matrix proteins that are type I collagen and the non-collagenous proteins such as ALP and osteocalcin. These proteins can hold the minerals as a template such as calcium and phosphate to form final hydroxyapatite phase by this way new bone formation can be started. Osteoclasts perform the bone remodeling as regulating the bone mass by using acid or enzymes which can dissolve the mineral phase and collagen. Polymerization of osteoclasts is controlled by integrin receptors which recognize Arg-Gly-Asp (RGD) sequence on matrix proteins. Finally, osteocytes which are derived from osteoblasts are the most abundant cells in the bone. They are responsible for the coordination between other bone cells as a response to hormonal and mechanical stimulates and maintenance of bone tissue. For example, as in Figure 2.3, micro damages can be detected by osteocytes and send signals to osteoclasts for bone remodeling. Besides, osteocytes make a response to mechanical loads then change bone mass by triggering other cells. As a conclusion, these cells work in a concert to maintain bone homeostasis [21, 2].

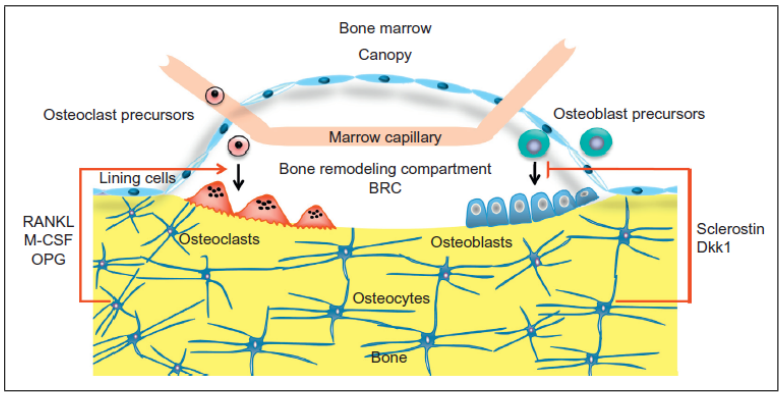

Figure 2.3 Remodelling of the bone tissue [21].

2.3 Bone Fractures and Healing

Bone fractures can occur due to many different reasons such as loss of anatomic continuity, mechanical instability, lower bone density or osteoporosis. Fracture types are classified as simple, comminuted and stress fractures. Bending or twisting force that causes breaking of the bones in two pieces by an injury which is shown in Figure 2.4. Although, in comminuted fractures, bone breaks into multiple pieces due to sudden and rapid forces like an accident or fall. Finally, in stress fractures, a low magnitude of the force is applied repeatedly which cause the collection of many micro-damages.

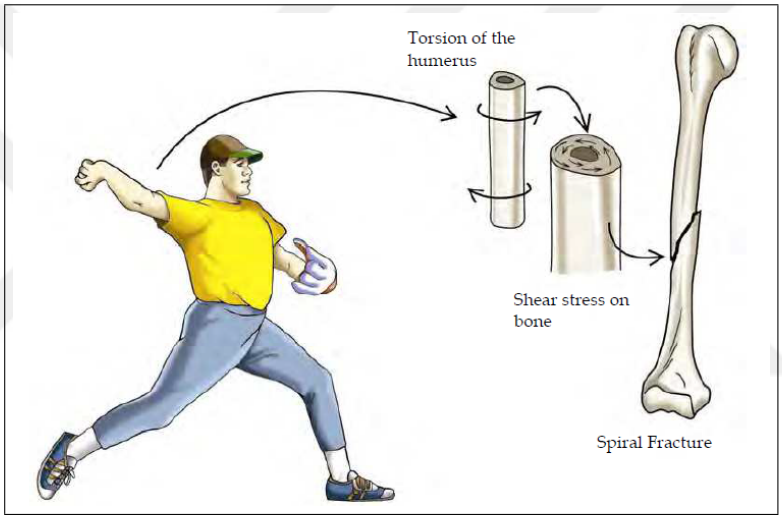

Figure 2.4 Bone fracture with torsion [24].

Healing of these fractures is also divided into two as primary and secondary bone healing. In primary healing, there is no motion which means a mechanically stable environment and no interfragmental gaps. Intracortical remodeling occurs between fragments as osteoclasts formed Haversian canals and osteoblasts fill the defect area. On the other hand, in secondary healing, there is a gap between fragments where intermediate phase callus is formed. In the beginning, this callus is a soft tissue but it becomes hard with the mineralization phase. Both intramembranous osteogenesis which controls the peripheral site of the fraction and endochondral osteogenesis which produces callus occur in secondary healing. In this time, woven bone is formed with less organized mineral phase as hydroxyapatite. This formation is important to give mechanical stability of the bone during healing [21, 25].

2.4 Bone Grafting Materials

Tissue engineering is a method for improving regeneration of tissues, cells that are damaged from different reasons such as fractures in bone tissue. In tissue engineering approach there are synthetic or natural materials which meet biological or mechanical needs to surrounding tissue. Tissue regeneration needs some basic components as a substrate which is essential for cell migration, cell proliferation, nutrient flow, and mechanical support; a signal which triggers the tissue regeneration via stimulate the cells and finally stem cells that receive the signals and then differentiate into host cell type in growth in the provided scaffold. However, in the body, every material cannot be served as a scaffold for tissue engineering because there are some criteria for healthy regeneration. Firstly, the scaffold should be biocompatible and mimic the extracellular matrix (ECM) that means it does not create an inflammatory response in the body. The porous structure is also a need for cellular migration, transport of nutrients, waste products and signals such as growth factors. The surface of scaffold should be suitable for cellular attachment and other interactions. Moreover, it has to provide mechanical support with anisotropic assembly until healthy tissue is formed. Additionally, it should have an appropriate degradation rate that allows the new tissue formation in place of scaffold [1].

In the literature, there are many different materials for bone tissue engineering as metals and alloys such as stainless steel, titanium; ceramics such as HA, TCP; polymers such as collagen, chitosan, PLGA and composites or nanocomposites such as HA/collagen or HA/PLLA. They are preferred for applications according to their advantages and disadvantages [2, 10].

2.4.1 Metals and Alloys

Metals like titanium or stainless steel are generally used in bone tissue engineering applications due to their higher elastic modulus which provides mechanical strength to the damaged area. However, this elastic modulus is nearly 5 times higher than nat-

ural bone, therefore, this cause stress on surrounded bone tissue which is known as stress-shielding. Metals also can corrode therefore induce an inflammatory response and they are dense and does not allow cellular migration or nutrient flow [2].

2.4.2 Ceramics

Ceramics have many varieties according to biological capabilities in addition to their biocompatibility and easiness to shape. They can be classified as bioinert (zirconia, alumina), bioactive (HA, bioglass) and bioresorbable (TCP). Alumina and zirconia have high hardness, biocompatibility, and corrosion resistance. However, they are brittle, have low resilience and their endurance decreases in the body over time. Besides, as shown in Figure 2.5HA has higher bioactivity which comes from the biological and chemical similarity of bone minerals with Ca/P ratio as 1.67. Bioactivity of HA means it interacts with the bone tissue and stimulates osteogenesis by promoting cellular adhesion, differentiation, and depositing of minerals on the surface. Nevertheless, its bioresorbability which means resorbing of the material during new tissue growth is low. TCP can handle this bioresorbability issue but the rate of resorption is uncertain and it has low mechanical properties [2, 4].

2.4.2.1 Calcium Phosphate Cements. In bone tissue engineering applications mimicking bone, biology is important to sustain biocompatibility. To achieve this, HA is the most used mineral both producing a composite material or cover other tough materials. Although HA constitutes nearly 70 of natural bone, when it is used as scaffold HA has some drawbacks such as low solubility, resorbability, an unstable structure in blood flow and problematic shaping [26, 7, 27]. Instead of directly using HA, calcium phosphate cements (CPCs) were started to use since the 1980s. This CPCs either resemble the mineral phase of the bone tissue or provide mechanical support, flexible structure to shape. Besides, Figure 2.6 and Figure 2.7 show that they can deposit as brushite or apatite form which has different Ca/P ratio. However, CPC which is deposited with 1.67 Ca/P ratio in an aqueous environment like water, PBS or

Physicochemical, mechanical, and biological properties of HA[a]	
Properties	Experimental data
Chemical composition	$Ca_{10}(PO_4)_6(OH)_2$
Ca/P molar	1.67
Crystal system	Hexagonal
Space group	$P6_3/m$
Cell dimensions (Å)	$a = b = 9.42$, $c = 6.88$
Young's modulus (GPa)	80–110
Elastic modulus (GPa)	114
Compressive strength (MPa)	400–900
Bending strength (MPa)	115–200
Density (g/cm^3)	3.16
Relative density (%)	95–99.5
Fracture toughness (MPa m$^{1/2}$)	0.7–1.2
Hardness (HV)	600
Decomposition temperature (°C)	>1000
Melting point (°C)	1614
Dielectric constant	7.40–10.47
Thermal conductivity (W/cm K)	0.013
Biocompatibility	High
Bioactivity	High
Biodegradation	Low
Cellular-compatibility	High
Osteoinduction	Nil
Osteoconduction	High

Figure 2.5 Properties of HA [2].

SBF and they are more closely resemble natural HA than sintered commercial HA [3, 28, 29]. Dissolution is the first step of setting reaction of CPCs. Calcium and phosphate ions are released in the solution and they start to precipitate in different forms according to the pH of the environment. The first CPC was investigated by Brown and Chow [30]. They use equimolar tetracalcium phosphate (TTCP; $Ca_4(PO_4)_2O$) and dicalcium phosphate dihydrate (DCPD; $CaH(PO_4).2H_2O$) or dicalcium phosphate anhydrous (DCPA; $CaHPO_4$).

An equimolar amount of TTCP and DCPA-DCPD make an injectable, molded self-setting paste with water and deposit as HA in an aqueous environment as shown in Eq. 2.1.

$$2Ca_4(PO_4)_2O + 2CaHPO_4 = Ca_{10}(PO_4)_6(OH)_2 \tag{2.1}$$

However, some differences between TTCP-DCPD and TTCP-DCPA phases. For example, TTCP-DCPD mixture has higher injectability, solubility and faster setting time

Ca/P molar ratio	Compounds and their typical abbreviations	Chemical formula	Solubility at 25 °C, −log(K_s)	Solubility at 25 °C, g/L	pH stability range in aqueous solutions at 25 °C
0.5	Monocalcium phosphate monohydrate (MCPM)	$Ca(H_2PO_4)_2\ H_2O$	1.14	~18	0.0–2.0
0.5	Monocalcium phosphate anhydrous (MCPA or MCP)	$Ca(H_2PO_4)_2$	1.14	~17	[c]
1.0	Dicalcium phosphate dihydrate (DCPD), mineral brushite	$CaHPO_4\ 2H_2O$	6.59	~0.088	2.0–6.0
1.0	Dicalcium phosphate anhydrous (DCPA or DCP), mineral monetite	$CaHPO_4$	6.90	~0.048	[c]
1.33	Octacalcium phosphate (OCP)	$Ca_8(HPO_4)_2(PO_4)_4\ 5H_2O$	96.6	~0.0081	5.5–7.0
1.5	α-Tricalcium phosphate (α-TCP)	α-$Ca_3(PO_4)_2$	25.5	~0.0025	[a]
1.5	β-Tricalcium phosphate (β-TCP)	β-$Ca_3(PO_4)_2$	28.9	~0.0005	[a]
1.2–2.2	Amorphous calcium phosphates (ACP)	$Ca_xH_y(PO_4)_z\ nH_2O$, n = 3–4.5; 15%–20% H_2O	[b]	[b]	~5–12 [d]
1.5–1.67	Calcium-deficient hydroxyapatite (CDHA or Ca-def HA) [e]	$Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$ ($0 < x < 1$)	~85	~0.0094	6.5–9.5
1.67	Hydroxyapatite (HA, HAp or OHAp)	$Ca_{10}(PO_4)_6(OH)_2$	116.8	~0.0003	9.5–12
1.67	Fluorapatite (FA or FAp)	$Ca_{10}(PO_4)_6F_2$	120.0	~0.0002	7–12
1.67	Oxyapatite (OA, OAp or OXA) [f]	$Ca_{10}(PO_4)_6O$	~69	~0.087	[a]
2.0	Tetracalcium phosphate (TTCP or TetCP), mineral hilgenstockite	$Ca_4(PO_4)_2O$	38–44	~0.0007	[a]

Figure 2.6 Calcium phosphate cements and their properties [3].

[3]. Although TTCP and DCPD are frequently used CaPs, there are also calcium sulfates such as calcium sulfate dihydrate (CSD; $CaSO_4.2H_2O$). CSD which has a similar crystal structure with DCPD has been used mostly in orthopedics due to its biocompatibility. Moreover, it increases the injectability of cement, accelerates the setting reaction. CSD also precipitate as HA in a TTCP and DCPD mixture in Na_2HPO_4 solution which is shown in Eq. 2.2 [31, 7, 32].

$$10CaSO_4.2H_2O + 6(HPO_4)^{2-} = Ca_{10}(PO_4)_6(OH)_2 + 8H^+ + 10(SO_4)^{2-} + 18H_2O \tag{2.2}$$

Nevertheless, it decreases the mechanical strength of the material because of its high resorption rate. To defeat this drawback CSD is combined with slowly degrading materials [33, 34]. Combination of CSD with TTCP and DCPD either solve resorption problem of it or lacking macroporosity in TTCP-DCPD based CPCs which is essential for cell ingrowth [32].

2.4.3 Polymers and Hydrogels

Polymers are biocompatible, flexible, moldable. Moreover, they have easy modification chance, diverse functional groups, controllable degradation rate and lightweight

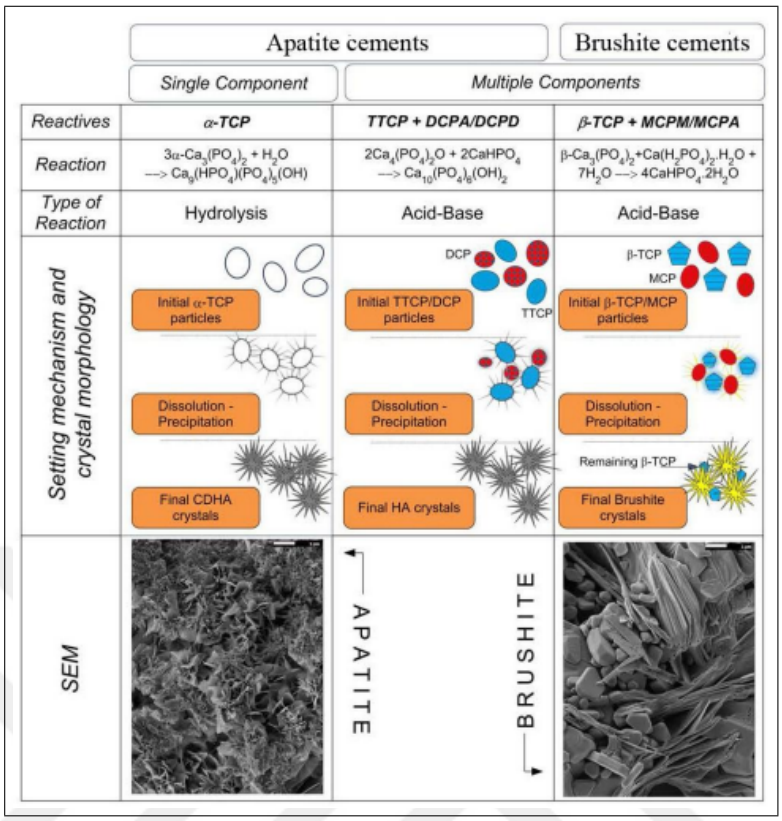

Figure 2.7 Formation of apatite and brushite cements [3].

that make them favorable materials for tissue engineering. They can be degraded both enzymatically and by hydrolysis at body pH. Polymers can be used from natural resources or synthetically according to the application. Synthetic or natural polymers can also be in hydrogel form which is 3D crosslinked hydrophiles. They can be crosslinked physically via hydrogen bonds, ionic interactions or dipolar interactions while chemical crosslinking is performed by covalent bonds. They swell under physiological conditions and do not dissolve. Besides, this swelling capacity can be tunable via physical or chemical changes such as pH, temperature or ionic strength. Their adjustable chemistry, hydrophilicity, and biocompatibility make them ideal biomaterials for diverse applications such as the food industry, water purification, drug delivery, lenses, wound healing applications[6, 4, 35].

2.4.3.1 Carboxymethyl Cellulose and Citric Acid. Cellulose is the most abundant plant-derived polymer in nature which contains β-1,4-glycosidic linkages between

its D-glucose monomers. However, cellulose has some drawbacks such as low solubility, stability, microbial resistance. There are cellulose derivatives to overcome these drawbacks one of them is a cellulose ether, carboxymethyl cellulose (CMC). CMC use in many applications such as food, paper, cosmetic, drug delivery, tissue engineering scaffold due to its hydrophilicity which comes from rich in hydroxyl and carboxyl groups on the surface, biocompatibility, non-toxicity and low cost [36, 37, 38, 39]. Also, these properties of CMC can be changed with its degree of substitution (DS). Although CMC is crosslinked chemically via esterification reaction to increase its swelling capability, many of the crosslinkers are toxic such as formaldehyde or glutaraldehyde. Therefore, the non-toxic and cheap alternative was developed in the literature with citric acid (CA). CA is mostly found in nature as a mineral and used in mainly food industry, drug industry, cleaning, and polishing. Carboxylic groups on CA provide binding sites for drugs by establishing ionic interactions or hydrogen bonding and by this way stabilize the drugs [35]. Moreover, cellulose which is crosslinked with CA achieves a thermally stable structure. Cyclic anhydride is produced when CA is heated. Then in the second step, this anhydride connects the two non-reacted carboxylic groups on cellulose/CMC molecules as esterification which is summarized in Figure 2.8 [5].Finally, with CMC and CA, a hydrogel matrix cage was performed in a water environment. Besides, this cage structure can be used in bone tissue engineering which provides immobilization of calcium phosphate particles thus strength of the material is increased. Also, CA is effective on HA formation/dissolution because citrate ions are already found in the bone structure. The main effect can be determined according to CA concentration in materials [7, 40].

2.4.3.2 Gelatin. Gelatin is a collagen derived natural polymer which is found in skin, bone or connective tissues. This biocompatible, biodegradable and non-immunogenic gelatin has two different types as A and B from hydrolysis of collagen. Gelatin A is obtained at low pH and mainly from skin tissue and gelatin type B is obtained at higher pH as the alkaline environment from bovine bones. Glutamic and aspartic acid residues are formed from glutamine and asparagine by this way in gelatin B carboxylic content is higher [42, 43]. Also, at lower pH values it can be cross-linked with

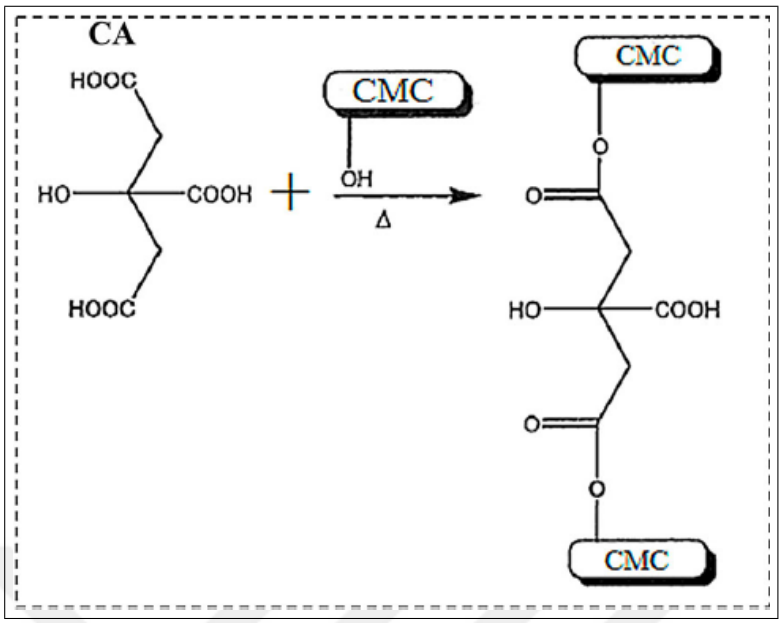

Figure 2.8 Esterification between CMC and CA [41].

CMC via hydroxyl and carboxyl groups [44]. At higher temperatures (>40Â°C) gelatin is in liquid form, however, at room temperatures, it undergoes gelation process by this way gelatin is used in biomedical applications as tissue engineering, microspheres, drug release [9]. In tissue engineering applications especially for bone regeneration, gelatin addition has many advantages. Firstly, gelatin is added in calcium phosphate cement to improve the cohesion of material, biocompatibility by mimicking the extracellular matrix of bone and mechanical strength. RGD sequence of gelatin increases cellular adhesion through the recognition process [45]. Besides, studies showed that gelatin increases the mechanical properties of CPCs due to the enhancement of the cohesion of CPCs in the polymer phase which brings about the homogenous distribution of load [9]. Therefore, gelatin can be a preferable material for bone tissue engineering with CMC-CA hydrogel [8, 46].

2.4.4 Composites

As seen in Figure 2.9,stress/strain graphic of bone grafting materials,mechanical properties of polymers or hydrogels are low and for ceramics these properties are enough but they have brittle structure. Besides, their biocompatibility is lower than polymers

because of possible toxic effect of their degradation for bone tissue regeneration when they used alone. Therefore, composite materials which consist of both polymers or hydrogels and ceramics aim to collect advantages and compensate for the disadvantages of each material. Their properties such as cellular attachment, mechanical properties, porosity, setting, degradation time can be controlled by different fabrication techniques and their final form is biocompatible, strong, flexible and reactive with their surfaces [2, 1, 47, 10].

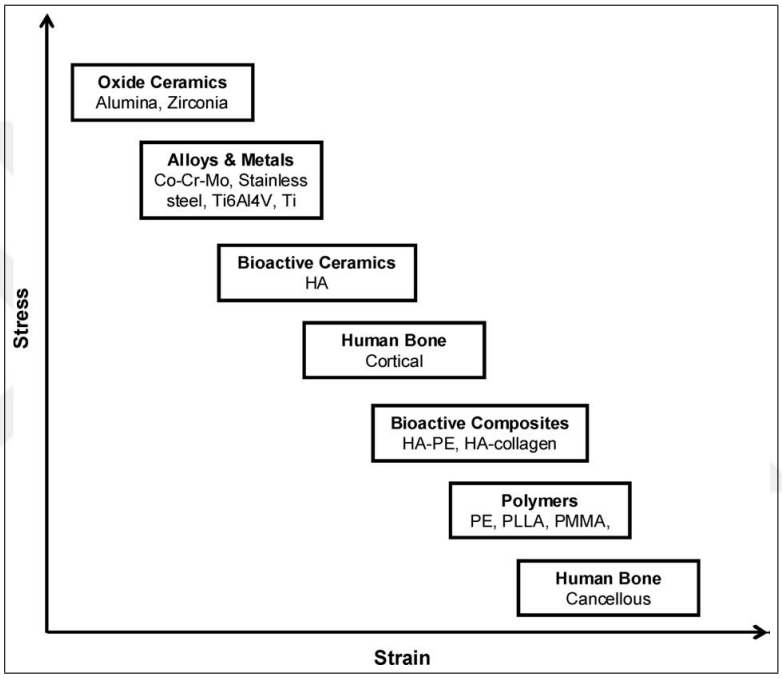

Figure 2.9 Comparison of stress-strain properties of bone grafting materials [2].

In the literature, for bone tissue engineering CPCs are mainly used with natural polymers which become suitable a combination with the biocompatibility of polymers such as chitosan, cellulose, PEG or gelatin [10]. They combined together as liquid and powder phase which is shown in Figure 2.10

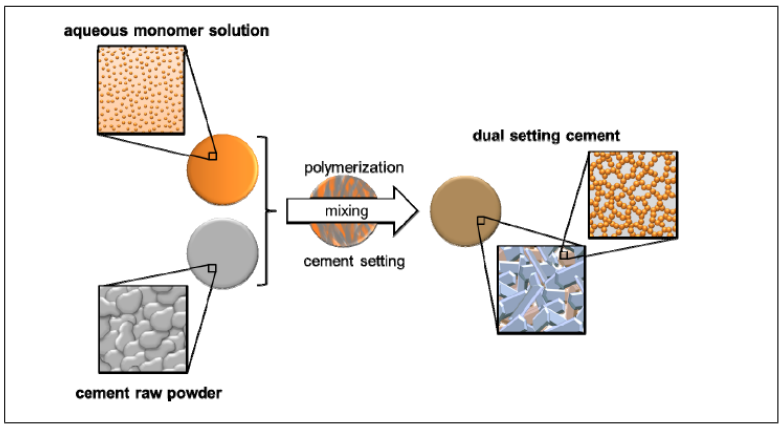

Figure 2.10 Preparation of the composites with liquid and powder phases [48].

3. MATERIALS and METHODS

3.1 Solid Phase Preparation

Firstly, TTCP was prepared according to previous literature by using a solid-state reaction as in Eq. 3.1;

$$2CaHPO_4 + 2CaCO_3 = Ca_4(PO_4)_2O + 2CO_2 + 3H_2O[7,\ 49,\ 50] \qquad (3.1)$$

Calcium hydrogen phosphate (DCPA, $CaHPO_4$, Mw: 136.06 g/mol) and calcium carbonate ($CaCO_3$, Mw: 100.09 g/mol, d: 2.93 g, $\leq 30\mu$m particle size) were mixed to produce TTCP. This mixture was heated by 10°C/min rate and when reach 1500 °C powder was held at this temperature for 6 hours. After that, the mixture was cooled with a rate of 10°C/min until it reached the room temperature. The final powder mixture was prepared with 76.65 wt% TTCP and 23.35 wt% calcium hydrogen phosphate dihydrate (DCPD, $CaH(PO_4).2H_2O$, Mw: 172.09 g/mol, d:2.31 g/mL). Calcium sulfate dihydrate (CSD, $CaSO_4.2H_2O$, Mw: 172.17 g/mol) was added to TTCP and DCPD mixture as 20 wt% of total TTCP-DCPD-CSD mixture. Powder phase was homogenized by blending of all powders for 15 min.

3.2 Liquid Phase Preparation

10 wt% of gelatin (from bovine skin, gel strength ~225 g Bloom, Type B) was dissolved in 2.5 w/v% of disodium hydrogen phosphate (Na_2HPO_4, Mw 141.96 g/mol, pH 8.7-9.3 at 25°C with 5% water) from Merck (Merck KGaA, Darmstadt, Germany)[51]. This solution was homogenized on a magnetic stirrer for 15min at 60 °C by closing the top of the beaker with foil. Then, 2 w/v% of sodium carboxymethyl cellulose (CMC, Mw 250.000 kDa, ds 0.9) was added on gelatin solution and kept on a magnetic stirrer for 45 min and the temperature was increased up to 90°C.After that, the temperature of the mixture was reduced to room temperature to add 20 wt% of

sodium citrate tribasic dihydrate ($C_6H_5Na_3O_7$, Mw 294.10 g/mol, mp: >300°C (lit.), pH 7.0 - 9.0 at 25°C). The solution was blended for 30 min. To remove residual water, the solution was put into the oven at 30 °C for 24h. For esterification reaction between citric acid and CMC, the temperature was raised up to 80 °C and kept for another 24h. Final hydrogel form is showed in Figure 3.1 [5, 6].

Figure 3.1 Hydrogel form after esterificaiton.

3.3 Hydrogel-Cement Composite Preparation

Composite was prepared by addition of powder phase on to esterified liquid phase gradually and mixed until it reached the homogenous chewing-gum like structure. Powder to liquid ratio was different for each sample as shown in Table 3.1. This mixture was molded into syringe whose tip was removed before. During molding, composites were carefully put into the syringe to obtain a homogenous composite with no air bubble formation and 2mm x 10mm dimensions. After that, composites were cured to set at 50°C for 72h which is showed in Figure 3.2.

Table 3.1
Powder to liquid ratios of composites.

Materials\Composites	Solid Phase 80% TTCP-DCPD (%76.65-%23.35) 20% CSD	Liquid Phase (2% w/v CMC- 10%wt Gel- 20%wt CA)
P62.5	62.5%	37.5%
P65	65%	35%
P67.5	67.5%	32.5%
P70	70%	30%

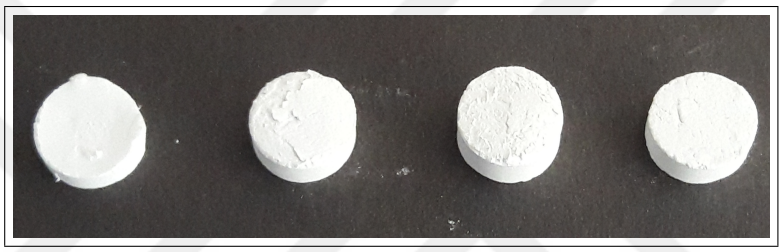

Figure 3.2 Composites after setting at 50° C.

3.4 Scanning Electron Microscopy (SEM) Analysis of Composites

Morphology of the composites were analyzed after the curing procedure and incubation in phosphate buffer saline (PBS, tablets, pH 7.4 at 25°C, Sigma Aldrich, Taufkirchen, Germany) to observe HA formation and changes in the composite structure for 14 days by using scanning electron microscopy (SEM; FEI-Philips XL30 ESEM-FEG) with 10 kV accelerating voltage by coating with gold-palladium before the experiment.

3.5 Swelling and Degradation Studies

Physical characteristics of composites were analyzed via swelling-degradation studies. Composites which were cured at 50 °C for 72h were put into 24 well plates

(n=4 for swelling and degradation) when they reached the room temperature. To start swelling and degradation studies samples were weighed to record their initial dry weight as W_i. For swelling studies,as in Figure 3.3,PBS solution was added on them as 1,5 ml for each well and incubated for 1, 8, 16, 24, 48 and 72 h at 37°C. After the certain incubation time, samples were put onto tissue paper and both sides were gently dried then their final weights were measured as W_f. Finally, by using W_i and W_f, %water uptake was calculated by the Eq. 3.2

$$\%WaterUptake = ((W_f - W_i)/(W_i)) \times 100 \tag{3.2}$$

At the same time for degradation studies, W_f was calculated at different steps as composites incubated in PBS solution for 1, 7, 14 and 21 days at 37°C. After that, the freeze-drying method was performed on composites which PBS solutions were discarded before. To determine the degradation percentage as % mass loss Eq. 3.3 was used.

$$\%MassLoss = ((W_i - W_f)/(W_i)) \times 100 \tag{3.3}$$

3.6 pH Studies

Measurements for pH of the composites (n=3) which again immersed in PBS solution for 1, 3, 5, 8, 16, 24, 48 and 72 hours at 37°C were performed. At the end of certain incubation times pH of the solutions was measured via pH meter (Thermo Scientific-Orion Star A211) and changes in pH values were observed.

3.7 Alamar Blue Assay

The cell viability was assessed with Alamar blue assay with a direct contact method. Counted cells as 10^5 per well were seeded on composites (n=3) in 10mm x 2mm disk form which were placed into 24 well plates after sterilization by UV for 30 min for both sides. Then, the assay was performed for 1, 3, 7 and 14 days of incubation

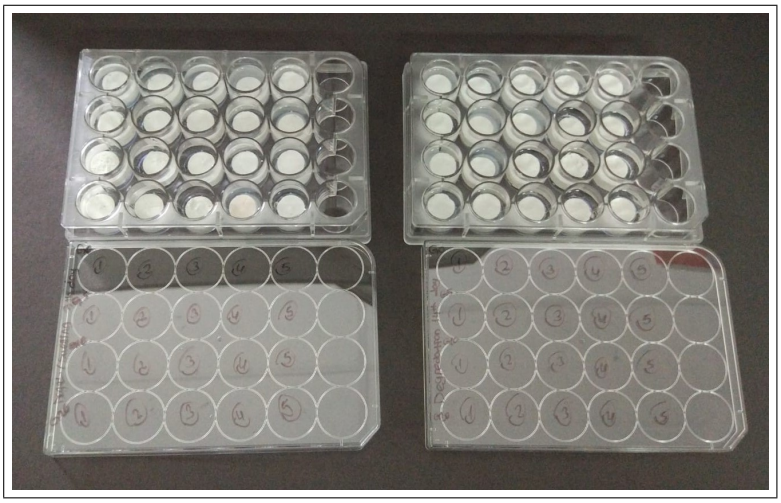

Figure 3.3 Composites which were incubated in PBS solution.

in PBS. At the end of incubation times old medium was completely discarded. Then, 450 μl fresh medium and 10% of Alamar blue were added as 50 μl. Cells and Alamar blue in plates were incubated at 37°C under 5% CO_2 for 3.5 hours under dark. After, 100 μl of solution from each well was transferred into 96 well plates which is indicated in Figure 3.4. Their absorbance at 570 and 595 nm was measured by using microplate reader (Bio-Rad Mark, Microplate Reader). % reduction of Alamar blue was calculated as the measurement of cell viability by the Eq. 3.4

$$\%Reductionof\,Alamar = \frac{E_1 \times A1(570) - E_2 \times A1(595)}{E_1 \times A2(570) - E_2 \times A2(595)} \tag{3.4}$$

Here;

E_1 is,117216, molar extinction coefficient of oxidized Alamar blue at 595nm.

A1 (570) is absorbance of the well at 570 nm.

E_2 is,80586, molar extinction coefficient of oxidized Alamar blue at 570 nm.

A1 (595) is absorbance of the well at 595 nm.

A2 (595) absorbance of negative controls at 595 nm.

A2 (570) absorbance of negative controls at 570 nm.

Negative control wells contained only medium and Alamar blue however in Tissue Culture Plate (TCP) wells also contained cells in addition to medium and Alamar blue. Both negative control and TCP wells did not have composites. Final calculations were made by taking TCP wells 100% viable.

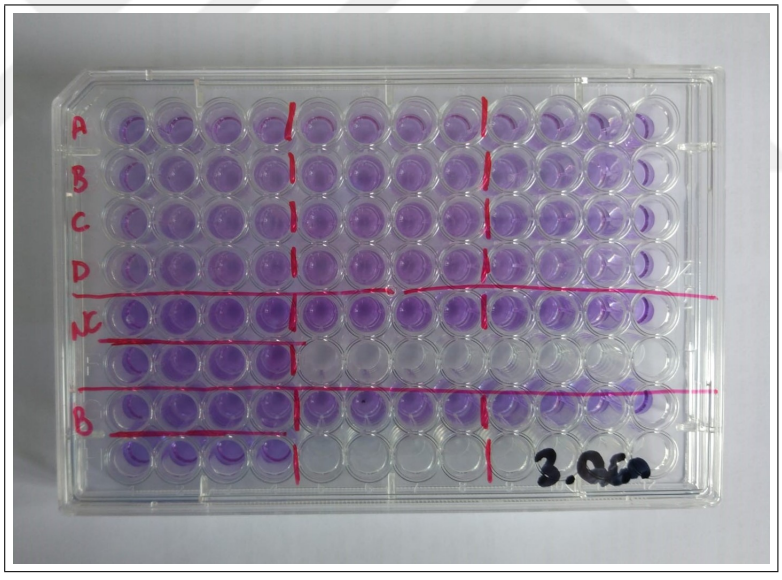

Figure 3.4 Alamar Blue assays of BMDMSCs.

3.8 In vitro Cell Adhesion

10^5 per well was seeded on composites in 10mm x 2mm disk form. Then, 1 ml of complete medium was added on them and incubated at 37°C, 5% CO_2 environment for 1 and 14 days. The medium was changed in every third day for 14 days of incubation.

After certain times, the medium was discarded and samples were washed with PBS for 2 times. For the fixation, 2.5% glutaraldehyde was added on the samples and incubated at +4°C for 3 hours. 30, 50, 70, 90, 100 % ethanol solutions were prepared and samples were incubated in them for 10 min respectively. Finally, 250 μl of hexamethyldisilazane was added on each sample and they were remained to dry until ethanol was completely evaporated. Morphology and adhesion properties of the cells were examined by using scanning electron microscopy (SEM; FEI-Philips XL30 ESEM-FEG) by coating with platinum.

3.9 Statistical Analysis

To calculate the reliability of the tests statistical analysis were applied by using SPSS for Windows software. The significance of the test calculated by applying one-way ANOVA with Tukey's post-test. Significance was accepted when $p \leq 0.05$ and signed with (*).

4. RESULTS

4.1 SEM Analysis of Composites

SEM images of composites after curing for 72h at 50 °C and incubation in PBS for 14 days at 37 °C are shown in Figure 4.1 and 4.2 respectively.

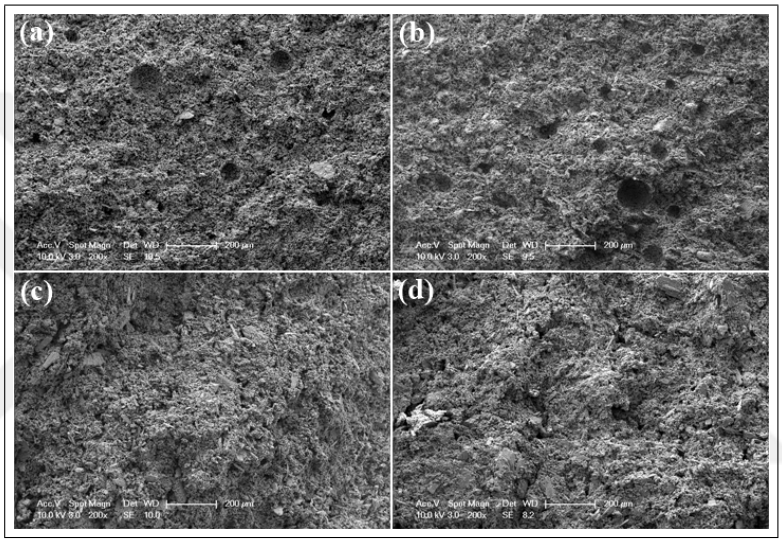

Figure 4.1 Composites after setting at 200μm.

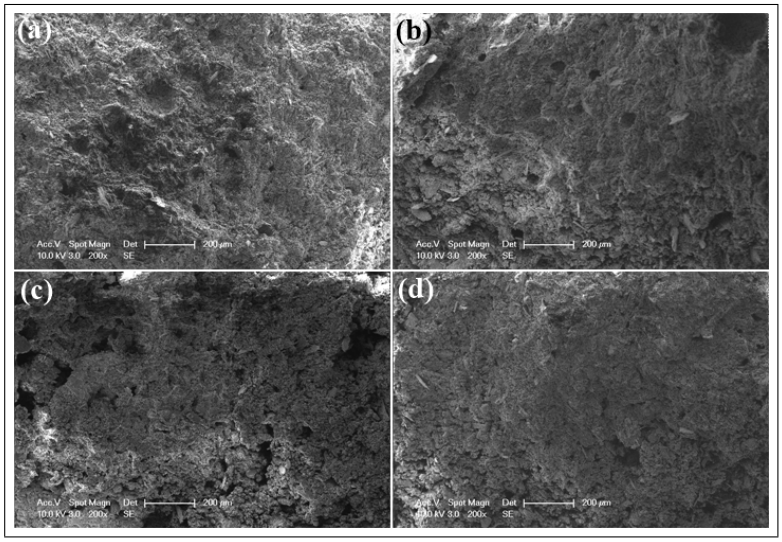

Figure 4.2 Composites after incubation in PBS solution for 14 days at 200μm.

According to images at 200 μm homogenous structure of CPCs which were embedded in the CMC-Gel hydrogel. Although P67.5 and P70 had a rough and less porous structure, micropores ($<$100 μm) could be observed in the structure of the P62.5 and P65. After 14 days of incubation in PBS, some of the pores in P62.5 and P65 were lost. Besides, disintegrations in the structure could be noticed for P67.5 and P70.

4.2 Swelling and Degradation Study

Figure 4.3 and 4.4 show the swelling and degradation characteristics of composites after certain times of incubation in PBS at 37C. Swelling which was shown as water uptake % was significantly increased between 1st and 8th hours ($p \leq 0.05$) for all composites. This increase was continued at the end of 48 h with slight changes. However, there were no significant changes between groups ($p > 0.05$). After 48 hours swelling tendency was lowered and reaching a plateau except for P65. At the end of 72 hours, water uptake % was found as 41.65 ± 0.51, 41.53 ± 0.33, 39.02 ± 0.51, 40.46 ± 0.68 for P62.5, P65, P67.5, and P70 respectively which means when powder ratio increased swelling degree decreased except for P70.

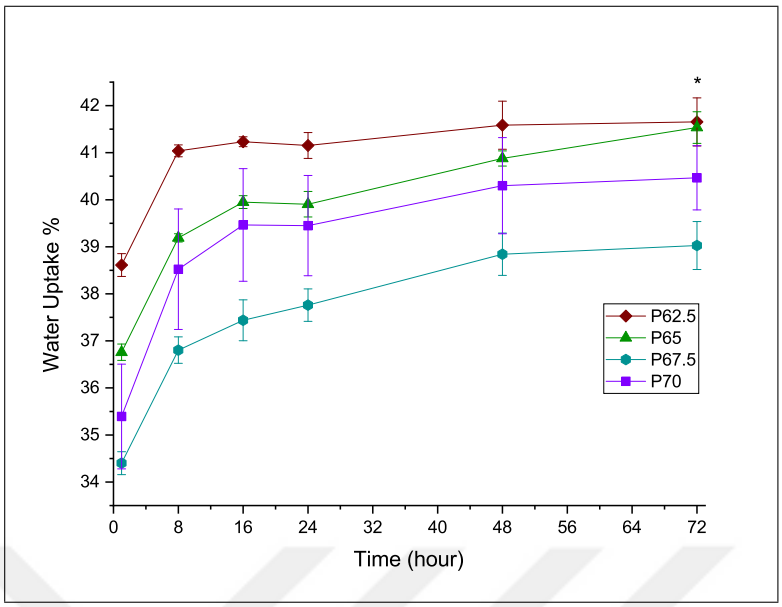

Figure 4.3 Swelling results of composites after 1, 8, 16, 24, 48 and 72h of incubation in PBS. Results are represented with standard errors.

According to degradation results, there were significant changes in the mass of the composites at the end of 21 days ($p \leq 0.05$) due to degradation of TTCP-DCPD-CSD powder phase in PBS. This mass loss kept the increased trend for all composites. Likewise, at the interval of 7 and 14 days for P70 mass loss % decreased which reached from 5.49 ± 0.71 to 4.62 ± 0.25. At the end of 21 days, it was found as 9.29 ± 0.42. Lowest mass loss was calculated for P67.5 as 6.46 ± 0.23. Figure 4.4 also showed that in homogenous composites increasing in powder phase cause lower mass loss via better integration. Nevertheless, the degradation study indicated that mass loss is a slow process for composites even after 21 days.

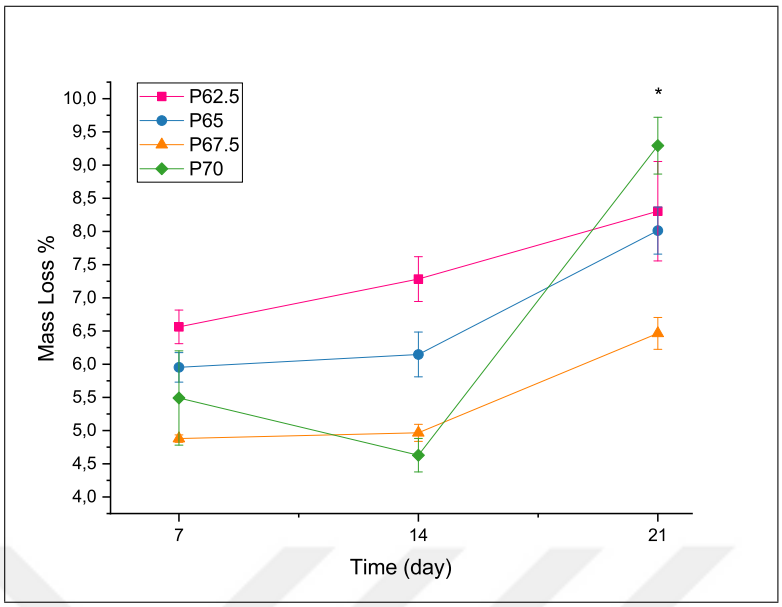

Figure 4.4 Degradation results of composites after 7, 14 and 21 days of incubation in PBS. Results are represented with standard errors.

4.3 pH Studies

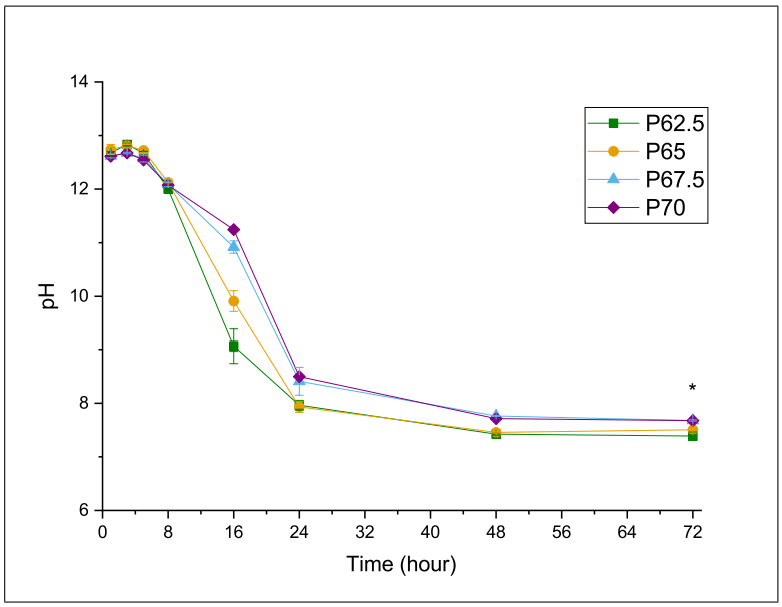

Figure 4.5 pH results of composites after after 1, 8, 16, 24, 48 and 72h incubation in PBS.Results are represented with standard errors.

Changes in the solution pH are demonstrated in Figure 4.5. At the first 8 hours, pH was around 12. After that, between 8-24 hours it diminished up to 7.9. There was a pH difference between groups at the time interval of 8-16 hours which was based on a higher powder ratio in composites induced higher pH values. After 16 hours, the pH of P62.5 was 9.06 ± 0.1 and P70 was 11.24 ± 0.11. At the end of 72 hours of incubation in PBS, pH of all solutions was stabilized to 7.3 which is close to physiological value.

4.4 Alamar Blue Assay

The effect of composites on the viability of BMDMSCs was measured via Alamar Blue assay for 1, 3, 7 and 14 days after incubating them at 37°C by the direct contact method. In Figure 4.6 % reduction of Alamar blue of the groups was shown by proportioning them with TCP plate which had 100% of viability. According to that, all groups had lower % reduction of Alamar blue than control. However, there was no significant difference between groups and days except P65. There was significant viability lost at 14th day compare to day 1 and day 3 ($p \leq 0.05$). P65 had $81.81 \pm 0.01\%$ and $73.35 \pm 0.06\%$ viability at day 1 and 3 respectively but it decreased to $66.46 \pm 0.015\%$ on day 14.

4.5 In vitro Cell Adhesion

In Figure 4.7adhesion of BMDMSCs is shown after cell fixation via SEM. According to scanning cell could adhere to the rough and hydrophilic surface of the composites. Although surface properties and biocompatibility of the composites were favorable for the cellular attachment, a few cells were observed probably due to the porous structure of the materials.

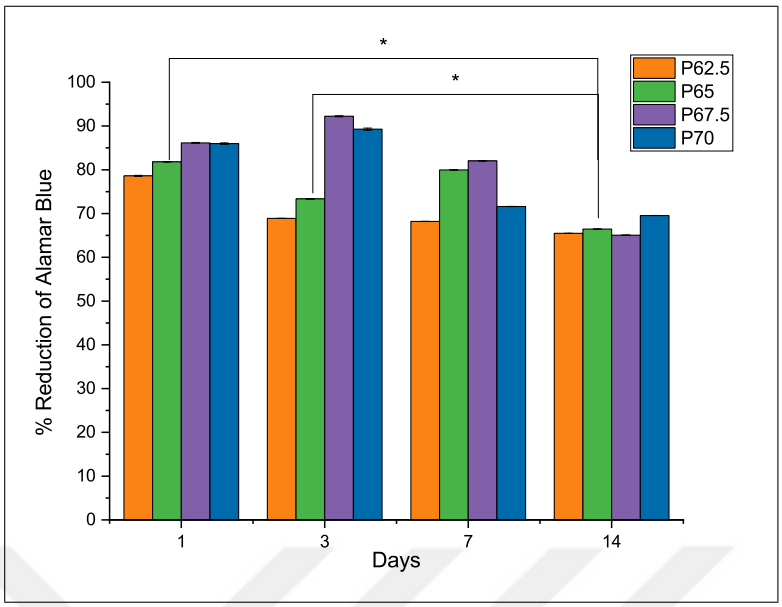

Figure 4.6 % Reduction of Alamar Blue represented as cell viability for composites after 1, 3, 7 and 14 days of incubation in PBS. Results showed with standard errors and $p \leq 0.05$ and signed with (*).

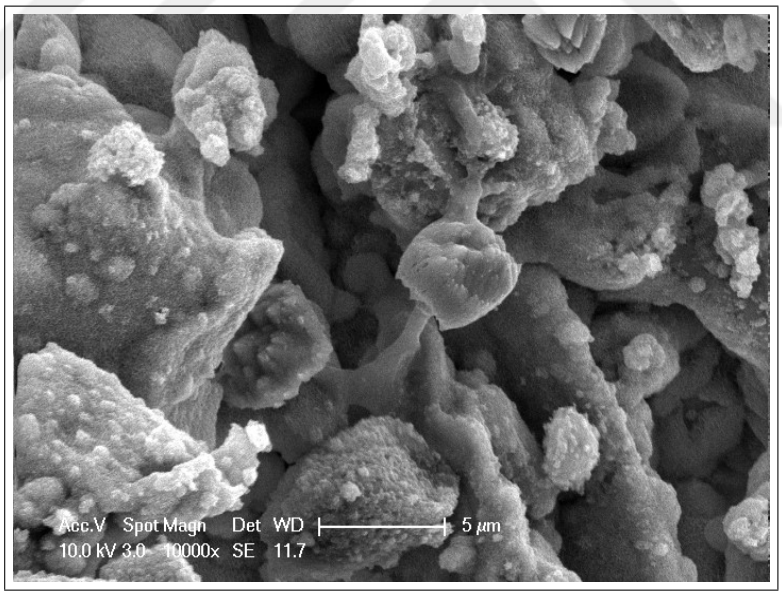

Figure 4.7 Attached cell on P62.5 composite at 5μm.

5. DISCUSSION

5.1 SEM Analysis of Composites

According to SEM results TTCP-DCPD-CSD powder phase was uniformly distributed in the hydrogel without any agglomeration. Combination of powder and liquid phase gave a rough surface to the composite which is important for the cellular adhesion, bioactivity, and proliferation [52]. The porous structure could be clearly observed in P62.5 and P65. These were micropores (<100 μm) which were introduced into the composite with the addition of the powder phase [53]. Also, with the increase of the powder phase, irregular cavities and disintegrations were seen mainly in P70 and also P67.5 [54]. After 14 days of incubation, some of the pores were disappeared. Probably, deposited HA crystals occupied the porous areas [55]. However, macropores were also observed in Figure 4.2b after PBS incubation because of the degradation of powder the phase. These macropores are important for the cellular attachment, adhesion and nutrient delivery during tissue regeneration[56].

5.2 Swelling and Degradation Study

Swelling is a critical feature for the biomaterials which helps to absorb nutrients and growth factors [57]. According to the swelling results, in the first 8-16 hours, higher water uptake was observed for composites which have a lower powder ratio. After that point, the swelling has reached a plateau for all composites. These results are accordance with the literature except for P70 [19, 46]. Swelling capacity is an important characteristics of the hydrogels, in here CMC-Gel hydrogel and micropores in composite caused swelling in the first 16h, cumulatively. The opposite trend between swelling and powder ratio arose due to decrease in hydrophilicity of hydrogel and water sorption capacity of the composite with the addition of the powder. When powder and liquid phases were mixed, interactions between polymer and CPCs increased there-

fore; polymer chains lost their elasticity which previously gave them to the swelling capability. Therefore, swelling capacity decreased [58]. However, P70 had a different swelling tendency from other composites. The reason might be improper mixing of this composite which was observed the during preparation stage. This disintegration caused more space between phases and also a higher swelling degree than P67.5.

Degradation or dissolution rate is important for the biomaterials for regulation of newly formed tissue and degraded scaffold. During tissue regeneration, dissolution of the scaffold can trigger the environment to the reconstruction of tissue by changing ion concentrations. For bone tissue, released Ca^{2+} and PO_4^{3-} ions can accelerate the bone tissue formation [59, 60]. Ion saturation of the microenvironment is increased which ends with nucleation of the HA crystals. Also, these precipitated crystals interact with other favored groups such as OH^- and CaO^- on the composites[33, 61]. This dissolution process can be mainly controlled by surface area, the acidity of the solution, temperature or solubility of the materials. After the setting process, CPCs had a slightly porous structure. They initially had only micropores therefore, they had less surface area. The combination with the polymer phase and further degradation provided macropores to composites which are important for bone regeneration [62, 61, 63, 64, 65]. The results were coherent with the literature which indicated that increase in the powder ratio cause a lower degradation degree because their porosity was decreased with increasing in the powder phase. Longer incubation time also caused a higher mass loss due to macropores which are produced during the degradation process[19, 33]. Only P70 had a different pattern for mass loss during incubation like in swelling studies. Disintegrations in composites could cause more deterioration and finally a higher mass loss than all the other composites.

5.3 pH Studies

Solution pH is critical for the formation of the apatite and biocompatibility therefore; it was investigated for the composites. Firstly, a very alkaline environment was observed by increasing in pH value to around 10-12. After further incubation,

it decreased to 7.3. This trend occurs mainly as a result of initial TTCP dissolution which created a basic environment. Then, both dissolution of DCPD-CSD and precipitation of Ca^{2+} and PO_4^{3-} ions on the surface as HA created a more acidic condition. H_3O^+ and H^+ ions were exchanged with Ca^{2+} ions and H^+ concentration was increased in the environment. Also OH^- ions were consumed during HA precipitation [63, 15, 66]. Although acidic pH can cause the inflammatory response in the body, the neutral environment was observed at the end of 72h for all composites that are close to physiological value [63]. Besides, initial alkaline condition and final neutral environment further triggered the HA formation [62, 34, 61]. These conclusions are valid for all composites because there was no significant difference between groups that means as pH aspect all composites have the same characteristics.

5.4 Alamar Blue Assay

Biocompatibility of the composites was measured by Alamar Blue assay for cellular viability. In this assay, TCP plates were used as a control. All composites had lower viability than TCP. Initial Ca^{2+} release which was also observed in pH results caused cell death. As known from literature calcium sulfates have low bioactivity. In the early stages, they cannot establish effective chemical interactions with their environment and excessive amount of Ca^{2+} ions caused change in the permeability of the mitochondrial membrane and so apoptosis of the cells [67, 33]. Although CPCs or calcium sulfate quickly dissolves under acidic conditions which are created by osteoclasts or other cells, integration with even a small amount of polymer contribute to the more cohesive structure and prevent washout of the composite and cell death [64, 68]. After 14 days there was viability loss for all composites. The potential reasons for that include increasing in debris concentration which is cytotoxic for the cells, and number of aged cell which lowered the cellular activities of young cells [67, 69]. However, significant viability decrease was observed in P65 on day 14. According to a previous study, HA crystals which are more precipitated into pores of P65 than the other composites decrease the surface area and energy of the composite. The produced HA crystals reduced the porosity which hindered the cellular growth [70, 71].

5.5 In vitro Cell Adhesion

Cellular attachment on the composites was observed with SEM. As known from previous studies porosity, hydrophilicity, roughness, charge distribution and crystal structure of the surface affect the cellular attachment, differentiation, and proliferation [59, 61]. Especially, rough and porous surfaces which provide higher surface area increase the protein adsorption [59, 61]. Also, the crystallinity of the CPCs can change the surface charge and pH that trigger the cell adhesion [61]. Cell adhesive proteins are generally negatively charged. CPCs have positively charged surfaces with their Ca^{2+} ions, therefore, interactions between cell and composite are promoted [61]. According to results, there are a few cells that could be observe from SEM images. Nevertheless, this result proved that the surface of the composite is suitable for the cell. Probably, a macroporous structure which was mainly formed by degradation and polymer phase combination caused more cell penetration into the composite. Hence, only few cells could be observed in SEM images [65].

6. CONCLUSION AND FURTHER STUDIES

In this study, a composite material was produced by using CMC and Gel as liquid phase and CPC as powder phase. According to results, composite had promising biocompatibility for bone tissue engineering which can be controlled by powder to liquid ratio. However, crystallization and activation of the composite in aqueous environment will be determined by Alkaline Phosphatase activity test (ALP) as a further study. Besides,the degradation time of the composites is very slow for local tissue regeneration. To beyond control of adaptability and resemblance of the composite for bone tissue, nanomaterials such as graphene oxide and carbon nanotube will be added into the composites.

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