INVESTIGATION OF BIOCOMPATIBILITY OF CALCIUM PHOSPHATE BASED MATERIALS AND CEMENTS

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ABSTRACT

INVESTIGATION OF BIOCOMPATIBILITY OF CALCIUM PHOSPHATE BASED MATERIALS AND CEMENTS

Calcium phosphate cements (CPCs) have been extensively investigated due to their excellent biocompatibility, osteoconductivity, potential resorbability in dentistry and orthopedics. They have numerous advantages over other calcium phosphate-based materials. The CPC precursor powders were prepared in the initial stage of this work. Tetracalcium phosphate (TTCP) powders coded as TTCP-1 (obtained from H_3PO_4 and $CaCO_3$) and TTCP-2 (obtained from $NH_4H_2PO_4$ and $CaCO_3$) were prepared by heat treatment of the calcium and phosphate source mixtures at 1350°C. Brushite powders were produced by aqueous chemical methods. A series of CPCs (HA cements) were prepared by using the TTCP-1 and brushite powders which were mixed with 0.2 M and 0.3 M phosphate buffer solutions at three different solid/liquid ratios (2.4, 2.7 and 3.2 g/ml) with three different HA initial seed contents (3%, 1.5% and 0.0 wt%).

The setting times of CPCs were determined to be in the 3.5-24 minute range. The phase structure and surface morphology of the cements and precursor powders were characterized by XRD and SEM. XRD analysis of powders revealed the presence of the characteristic TTCP and brushite peaks. XRD analysis also indicated that all cement samples were composed by HA phase with different crystallinity and other phases were not detected. Rod and plate-like hydroxyapatite crystals were observed in the SEM micrographs of all CPCs.

Cytotoxicity testing was performed using the MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide) assay to determine the number of viable cells in the presence of powders and HA cements. Cytotoxicity results indicated that brushite powder caused sharp decreases in cell viability at the end of 24, 48 and 72 hours at all powder extract concentrations. TTCP-1 and TTCP-2 powders unlike brushite had no toxic effect with cell viability values over 74 %. Almost all CPCs prepared in this work had no cytotoxic effects.

ÖZET

KALSİYUM FOSFAT BAZLI MALZEME VE ÇİMENTOLARIN BİYOUYUMLULUĞUNUN İNCELENMESİ

Kalsiyum fosfat çimentoları (CPCs), dişçilik ve ortopedik uygulamalardaki mükemmel biyouyumlulukları, osteo iletkenlikleri, potansiyel özümlenebilirlikleri nedeniyle kapsamlı olarak incelenmektedir. CPC'ler diğer kalsiyum fosfat bazlı malzemelere göre bir dizi avantaja sahiptirler. Bu çalışmanın ilk aşamasında kalsiyum fosfat çimentolarının öncül tozları hazırlanmıştır. TTCP-1 (H_3PO_4 ve $CaCO_3$ dan hazırlanan) ve TTCP-2 ($NH_4H_2PO_4$ ve $CaCO_3$ dan hazırlanan) olarak kodlanan tetra kalsiyum fosfat tozları, kalsiyum ve fosfat kaynaklarının karışımlarının 1350°C de ısıl işlem uygulanması ile hazırlanmıştır. Bruşit tozu sulu kimyasal metod ile hazırlanmıştır. Bir dizi CPC'ler (HA çimentoları) çeşitli hidroksiapatit başlangıç çekirdek derişimlerinde (3%, 1,5% ve 0.0% ağırlık bazında), üç farklı katı sıvı oranında (2,4, 2,7 ve 3,2 mL/g), TTCP-1 ve bruşit tozlarının 0,2 ve 0,3 molar fosfat tamponu solüsyonu ile karıştırılarak hazırlanmıştır.

CPC'lerin katılaşma zamanları 3.5 ile 24 dakika aralığında belirlenmiştir. Çimentoların ve öncül tozların faz yapısı ve yüzey morfolojisi XRD ve SEM ile karakterize edilmiştir. Tozların XRD analizleri, tetrakalsiyum fosfat ve bruşitin karakteristik piklerinin varlığını açığa çıkarmıştır. Benzer şekilde XRD analizleri tüm çimento örneklerinin HA fazında değişik kristaliniteye sahip olduğunu ve başka fazların görülmediğine işaret etmiştir. Tüm hidroksiapatit çimentolarının SEM incelemelerinde çubuğa-plakaya benzer hidroksiapatit kristalleri görülmüştür.

Sitotoksite testleri hidroksiapatit çimentoları ve tozların varlığında canlı hücrelerin sayısını belirlemek için MTT (3-(4,5-dimetiltiazol-2-yl) 2,5-difenil tetrazolyum bromit) analizi ile gerçekleştirilmiştir. Sitotoksisite sonuçları, bruşit tozunun tüm toz özüt derişimlerinde 24, 48 ve 72. saatlerin sonunda hücre canlılığı üzerinde belirgin bir düşüşe sebep olduğuna işaret etmiştir. Bruşitin aksine TTCP-1 ve TTCP-2 tozları kontrol grubu ile karşılaştırıldığı zaman, %74 ve üzerindeki hücre canlılığı değerleri ile toksik etkiye sahip değildir. Bu çalışmada hazırlanan neredeyse tüm kalsiyum fosfat çimentolarının sitotoksik etkiye sahip olmadığı gözlenmiştir.

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CHAPTER 1

INTRODUCTION

Calcium phosphate cements (CPC) are extensively used as bone substitute biomaterials in orthopedics and dentistry for their high biocompatibility. They can be simply shaped to adapt to the gaps of defect surfaces at a microscopic level and exhibit osteoconductivity (Guo et al. 2005).

A large number of CPC compositions by using various calcium phosphate powders/phases have been formulated during the last decade. Their characteristics and *in vitro* and *in vivo* properties have been investigated because of their excellent biological properties, potential resorbability, molding capabilities, and easy manipulation. In general, there are two types of CPC, apatite cements and brushite cements according to the final product. Hydroxyapatite [HA - $Ca_{10}(PO_4)_6(OH)_2$] is the most often studied, clinically tested and used calcium phosphate phase as synthetic bioceramic materials among all the other calcium phosphate (CaP) compounds (Berzina and Borodajenko, 1996). Hydroxyapatite (HA) constitute the mineral components of hard tissues such as bones and teeth. Hydroxyapatite has a high compatibility for use as a substitute for natural hard tissues. Self-setting calcium phosphate cements commonly with a final hydroxyapatite phase structure currently sets within 30 minutes *in situ* when calcium phosphate cements are used as an implanted artificial hard tissue (Patel, 2011). This cement system thus is very convenient to use in clinical aplications. Brushite cement however has lower mechanical strength but a faster biodegradability than the apatite cement.

Apatite cement is a bioactive and biodegradable grafting material consisting of a mixture of powder and liquid, which when mixed, sets primarily as hydroxyapatite. The material can potentially be replaced with bone after a period of time, it could retain the short-term biological advantages of hydroxyapatite (Komath and Varma, 2003).

The setting reactions of calcium phosphate cements (CPC) usually occur via acid–base reactions between several calcium orthophosphate combinations, which set *in situ* in the presence of an aqueous phase. The proportion of solid to liquid or the powder-to-liquid (P/L) ratio is a very important characteristic since it determines both

bioresorbability and rheological properties. Cement formation is based on the difference in pH-dependent solubilities of calcium phosphates. Above pH 4.2 HA is the product, whereas acidic mixtures (pH < 4.2) form brushite (Barralet et al. 2004).

Biocompatibility is defined as the ability of a material to perform with a convenient host response in a specific application. Depending on the material composition, different tissue responses may result. If the tested material is nontoxic and biologically active then it will integrate with the surrounding tissue. However, if it is biologically inactive, it may be encapsulated by a fibrous capsule. On the other hand, if the material is toxic, rejection and localized death of the surrounding tissue can occur (Zaazou et al. 2013).

Biomaterials are defined as natural or synthetic materials used to replace parts of a living system or to function in intimate contact with living tissues (Dorozhkin, 2011a). For any biomaterial used as bone implants, *in vivo* dissolution of the material is a significant issue. Since, macrophages or giant cells may probably move to suppress any foreign material, when this material is implanted into the body. Therefore, through the immersion of these materials into simulated body fluids *in vitro* have been used to estimate *in vivo* bone bonding properties of varied materials. (Barralet et al. 2004).

Medical devices need to be tested for their biocompatibility before their contact with the human body because of their probable negative effects. Nowadays harmonized standards issued by the international bodies for standardization (ISO) gives the assurance about the essential requisites of medical devices, including biocompatibility. Cytoxicity tests are recommended for all medical devices to perform a rapid evaluation and to eliminate toxic materials prior to animal testing (Pearce et al. 2007).

Many *in vitro* assays are often defined to provide the evaluation of biocompatibility, in many cases according to ISO 10993, which describes the topics of evaluation and testing methods relevant for the biological evaluation of medical devices (Rodrigues et al. 2012). Cytotoxicity tests are generally based on the lysis of cells (cell death), the inhibition of cell growth, or other effects on the cells that the medical devices, materials and/or their extracts exert, with the use of cell culture techniques. *In vitro* cytotoxic assays are simple, reproducible, cost-effective, relevant and suitable for the evaluation of basic biological aspects relating to biocompatibility. A very wide number of assay techniques can be used to evaluate *in vitro* cytotoxicity. The most common assay for the evaluation of cytotoxicity is the dimethyl-thiazol-diphenyl tetrazolium bromid (MTT) test which demonstrates some significant advantages such as

simplicity, rapidity and precision (Deus et al. 2005). This test specifically evaluates an enzyme function, as the yellow tetrazolium salts are reduced to water-insoluble purple-blue formazan crystals by mitochondrial dehydrogenases. These blue formazan crystals precipitate in the cytosol and then solubilized after the induction of cellular lysis by a surfactant, enabling the absorbance to be red at 540 nm (Rodrigues et al. 2012).

The reported research on the effects of buffer strength, solid/liquid ratio and the HA seed content utilized on the in vitro cytotoxicity of apatitic bone cements is very limited to the best of our knowledge. The purpose of this study was to investigate cytotoxicity of HA cements prepared using tetracalcium phosphate and brushite powders. TCCP and brushite powders were first chemically synthesized and characterized. Cements were prepared by mixing the powders with sodium phosphate buffer with various buffer strength and pH values. Setting times and cytotoxicity of the prepared cements were finally evaluated.

CHAPTER 2

BONE AND CALCIUM PHOSPHATE CEMENTS

2.1. Bone

Bone is a ceramic-organic composite that consists of compact (cortical) bone and the cancellous (trabecular or spongy) bone. Compact bone makes up the hard outer surfaces of the bones, and it is usually heavy and dense compared to the spongy bone. Spongy bone is very porous and less dense than compact bone. Cancellous bone is typically surrounded by compact bone. Both cortical and trabecular bone are composed of osteons. The main constituents of bone are collagen (20 wt. %), calcium phosphate (69 wt. %), and water (9 wt. %). Additionally, other organic materials, such as proteins, polysaccharides, and lipids are also present in small quantities. (Pighinelli and Kucharska, 2013). An overall composition of bone is given in Table 2.1.

Table 2.1. Composition of bone (Source: Dorozhkin, 2009a).

Inorganic Phases	wt %	Bioorganic Phases	wt %
Calcium Phosphates (Biological apatite)	~ 60	Collagen type I	~ 20
Water	~ 9	None – collagenous proteins	~ 3
Carbonates	~ 4	Primary bone cells	Balance
Citrates	~ 0.9	Other traces	Balance
Sodium	~ 0.7		
Magnesium	~ 0.5		
Other traces	Balance		

Cortical bone consists of cylindrical structures known as osteons and Haversian systems approximately 10-200 µm wide at their base and is formed from by cylindrical lamellae surrounding the Haversian canal. Haversian systems are in cylindrical shape and form a branching network within the cortical bone. Blood vessels are located in the center of each osteon inside the Haversian canals. The metabolic substances can be transported by the intercommunicating systems of canaliculi, lacunae and Volkman's

canals. The walls of Haversian systems are formed from of concentric lamellae. Collagen can be considered as the matrix of small microfibers. It is difficult to observe collagen fibers in the matrix. At the moleculer level, the HA crystals are about 40–60 nm long, 20 nm wide, and 1.5–5 nm thick. Hydroxyapatite crystals are oriented along the collagen fibers. These mineralized collagen fibers are arranged into the lamellar sheets with a thickness of 3-7 µm. Four to twenty lamellae occur as a concentric ring around the Haversian canal and form an osteon. The hiearchical levels and structural organization of a human bone are shown in Figure 2.1 (Suchanek and Yoshimura, 1998).

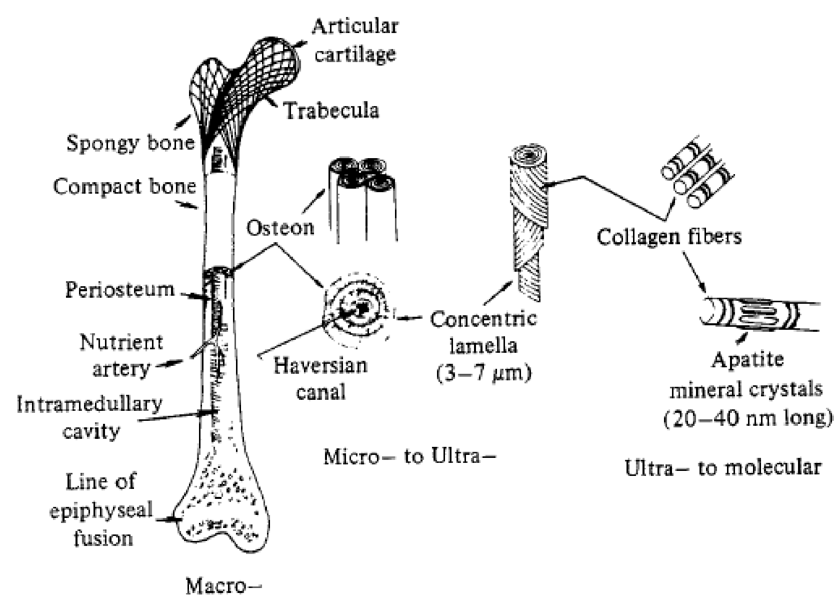


Figure 2.1. Hiearchical levels and structural organization of human long bone (Source: Suchanek and Yoshimura, 1998).

The major component of the bone mineral is crystalline hydroxyapatite[HA,$Ca_{10}(PO_4)_6(OH)_2$]. HA forms about a quarter of the volume and half of the mass of normal adult bone.

The calcium and phosphorus (inorganic phosphate) components of these crystals are supplied by the blood plasma. Amorphous calcium phosphate matures through several intermediate stages to form hydroxyapatite. A highly organized complex microstructure of proteins and mineral, primarily collagen and hydroxyapatite eventually occurs. The structural durability of HA is the source of the adequate mechanical functions of the skeleton (Kini and Nandeesh, 2012).

2.2. Biomaterials and Bone Grafts

A biomaterial is defined as non-viable material used in the production of medical devices in order to interact with biological systems. Biomedical materials can be used to treat, improve, replace any tissue, organ, or function of the body for any period of time. These materials are natural or synthetic in origin (Salgado et al. 2011). Many materials have been used as potential biomaterials such as surgical glues, stents, external fixation devices, wound dressing, and dental materials. The materials used in orthopedics and dental applications are components of structural implants or used to repair bony defects. These implants can be metallic, polymeric, ceramic or composites. The selection of materials used for implants is very important in terms of their function in the body (Mihov and Katerska, 2010).

Calcium phosphate-based biomaterials, specifically hydroxyapatite ceramics have generated a great deal of interest in relation to hard tissue applications basically due to their bioactivity. Calcium phosphate cements (CPCs) are also very important for bone tissue engineering. Various studies conducted by calcium phosphate cements (CPCs) have shown that they can induce tissue regeneration and also they are greatly biocompatible and osteoconductive materials (Pignatello, 2011).

Synthetic HA can be fabricated in different shapes as porous or solid blocks or granules. HA crystals are subjected to heat treatment (sintered) between 700 and 1300°C to form a highly crystalline structure (Moore et al. 2001).

Bone grafts are often required to provide support, to fill voids, and to repair skeletal defects which may be traumatic or non-traumatic in origin (Nandi et al. 2010). There are various bone graft types such as autografts, allografts, synthetic grafts, and others which are fully characterized and applied clinically.

Autogenous bone grafts are made from the patient's own bone. They are considered as the gold standard for bone graft materials supplying osteoinductive growth factors, osteogenic cells and being a structural scaffold. On the other hand, there are some disadvantages such as donor site morbidity, second surgery and limited availability.

Allogenous bone grafts come from the bone tissues of an individual of the same species. These are the next best alternative at present; however, disadvantages of allograft bone are minor immunogenic rejection and risk of disease transmission.

Xenogenous bone grafts are also known as xenografts and are obtained from individuals of different species (for example bovine bone). They can cause allergic and immunogenic reactions. Alloplastic bone grafts are synthetic materials manufactured in different shapes, textures, sizes and compositions. This category includes metallic grafts, calcium phosphate ceramics (hydroxyapatite and tricalcium phosphate), calcium carbonate, calcium sulphate, Hard Tissue Replacement (HTR) polymers and bioglass (Salgado et al. 2011).

The calcium phosphate based grafts have both osteointegrative and osteoconductive properties. Osteointegration results from the formation of a layer of HA shortly after implantation. The Ca^{+2} and PO_4^{-3} ions required to establish this layer are derived from the implant and surrounding bone. Calcium phosphate based grafts have an excellent record of biocompatability with no reports of systemic toxicity or foreign body reactions (Moore et al. 2001). A surgical procedure for filling of the bone defect in a sheep is shown in Figure 2.2.

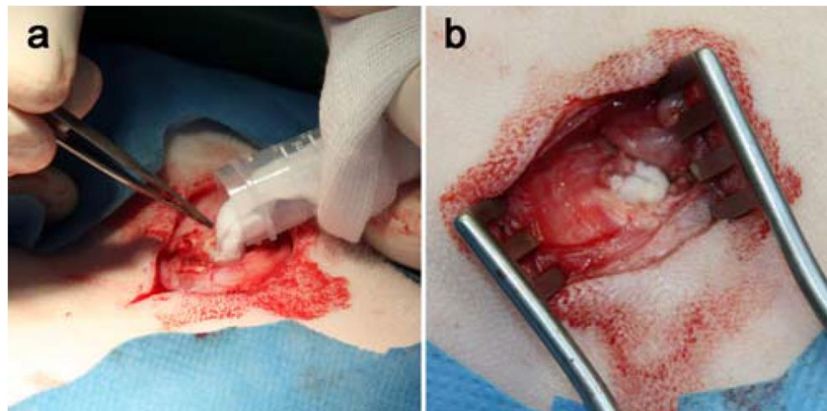


Figure 2.2. A surgical procedure for filling of bone defects
(Source: Schneider et al. 2011).

2.3. Calcium Phosphate Bone Cements

Calcium phosphate cements (CPCs) have been used increasingly as bone graft substitutes in dental and medical applications in terms of unique *in vivo* properties, such as, the similarity to hard tissues, bioactivity, biocompatibility, slow resorption and replacement by new bone as a result of the cellular action of bone cells (osteoclasts and osteoblasts) (Takagi and Chow, 2001). The current commercial CPCs however have

some limitations due to their poor mechanical properties, slow *in vivo* biodegradation and the absence of macroporosity (Julien et al. 2007). CaP based bone graft substitutes can be used in different forms such as granules, porous blocks, cements, putties, sponges/foams, or strips/membranes (Bohner et al. 2013).

Calcium phosphate cements are formed by mixing a liquid and a powder phase in paste form. The cement sets in about 30 min after being implanted within the body. The cement develops sufficient mechanical strength for repairing hard tissues defects. There are two main types of cements with respect to the final product as apatite and brushite cements. Both apatite and brushite cements can be applied for bone tissue engineering purposes. Despite the fact that brushite cement has a faster biodegradability, it has a lower mechanical strength than the apatite cement (Pignatello, 2011).

The principle of the formation CPCs is based on dissolution/reprecipitation of acidic and basic calcium phosphate compounds upon wetting with an aqueous medium at appropriate pH and temperature. The powders dissolve in the liquid and this liquid then becomes supersaturated where the nucleation and the precipitation of at least one calcium phosphate occurs. Most of the calcium phosphate cements have an apatitic precipitate. The cement body gains strength by the entanglement of the crystals of the precipitate (Driessens et al. 2002).

The weight ratio of solid to liquid (S/L) is a very important characteristic because it determines both bioresorbability and rheological properties (Dorozhkin, 2009a). Mixing of calcium phosphate powders and the liquid phase with suitable proportion gives a self-setting mass. The setting time can be adjusted by adding manipulator compounds (physiologically acceptable phosphates) to the wetting medium. The phase of the set mass depends on the Ca–P ratio of the mix, for example when the Ca/P ratio is adjusted to 1.67, the final phase will be hydroxyapatite (Komath et al. 2003). Varius calcium phosphate phases are given Table 2. 2.

Table 2.2. Calcium phosphate phases with their respective Ca/ P atomic ratios (Source: Dorozhkin, 2009a).

Name	Ca/P Mol Ratio	Formula	Symbol
Tetracalcium phosphate	2	$Ca_4(PO_4)_2O$	TTCP
Hydroxyapatite	1.67	$Ca_{10}(PO_4)_6(OH)_2$	HA
Tricalcium phosphate	1.5	$Ca_3(PO)_2$	TCP
Octacalcium phosphate	1.33	$Ca_8H_2(PO_4)_6.5H_2O$	OCP
Dicalcium phosphate anhydrous	1	$CaHPO_4$	DCPA
Dicalcium phosphate dihydrate	1	$CaHPO_4.2H_2O$	DCPD
Calcium dihydrogen phosphate	0.5	$Ca(H_2PO_4)2H_2O$	MCP
Calcium pyrophosphate	1	$Ca_2P_2O_7$	CPP
Precipitated hydroxyapatite "tricalcium phpsphate"	1.5-1.67	$Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$	PHA
Heptacalcium phosphate	0.7	$Ca_7(P_5O_{16})_2$	HCP

2.4. Hydroxyapatite

Hydroxyapatite ($Ca_{10}(PO_4)_6OH_2$), also referred to as calcium hydroxyapatite belongs to the apatite group. It has a specific composition and a crystallographic structure, with Ca/P stoichiometric ratio of 1.67 (Nathanael et al. 2013).

Hydroxyapatite has a hexagonal symmetry and unit cell lattice parameters a = 0.942 nm and c = 0.687 nm. This space group is characterized by a sixfold c- axis perpendicular to three equivalent a-axis (a_1,a_2,a_3) at angles 120° to each other with a mirror plane (Hench and Wilson, 1993). The atomic structure of HA and its projection along the *c* axis are shown in Fig. 2.3. The Ca atoms are located in two positions: six atoms per unit cell are in position Ca (II) and four atoms are in position Ca(I). Ca(I) is located on the threefold axis and is coordinated by nine oxygens of phosphate groups. The Ca(II) atoms constitutes equilateral triangles. Within each triangle, a fluorine atom positions centered on the hexagonal axis; the OH groups reside in an off-center position (Orlovskii et al. 2002).

The apatite in bone mineral consist of small platelet-like crystals of just 2 to 4 nm in thickness, 25 nm in width, and 50 nm in length (Dorozhkin, 2009b). Bone mineral non-stoichiometry is primarily due to the presence of divalent ions, such as CO_3^{-2} and HPO_4^{-2}, which are substituted for the trivalent PO_4^{-3} ions. Substitutions by CO_3^{-2} and HPO_4^{-2} ions result in a change of the Ca/P ratio which may alter between 1.50 to 1.70, based on the age and the bone site (Stanciu et al. 2007).

Biological hydroxyapatite also contains other impurity ions as Cl, Mg, Na, K, and F and trace elements like Sr and Zn. Substitutions in the apatite structure affect its properties such as lattice parameters, spectral properties, crystal size and morphology thereby affecting its chemical stability (solubility) and thermal stability. The degree of the effect depends on the size and the amount of the substituting ion (LeGeros, 2008).

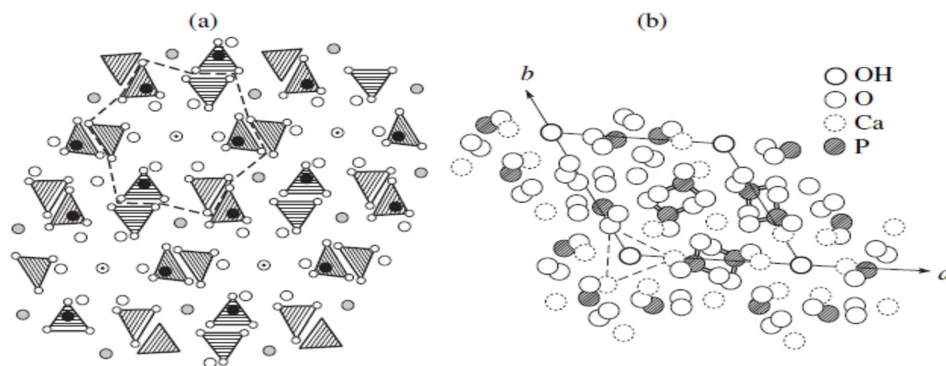


Figure 2.3. (a) Atomic structure of HA and (b) its projection along the c axis
(Source: Orlovskii et al. 2002).

The ceramic materials based on hydroxyapatite (HA) have shown the greatest potential for bone substitution in terms of biocompatibility. HA can improve tight bonding with bone tissue, exhibits osteoconductive behavior, is stable toward bioresorption, has no adverse effects on the human body. All apatite CPCs are viscous, easily moldable (Orlovskii et al., 2002). On the other hand, based on the low reliability, especially in the wet environments HA ceramics can not presently be used for heavy load bearing applications, like artificial teeth or bones (Nayak, 2010).

Various methods such as, precipitation technique, sol-gel approach, hydrothermal technique, multiple emulsion technique, biomimetic deposition technique, electrodeposition technique and etc. have been used for the synthesis of HA (Nayak, 2010).

The most popular and widely used technique for the synthesis of HA is precipitation. This technique is also named as wet precipitation or chemical precipitation or aqueous precipitation (Nayak, 2010). HA powder can be prepared by wet chemical methods, by solid-state reactions, and by hydrothermal treatment. All apatite CPC formulations have precipitated hydroxyapatite (PHA) as the end-product of the reaction, and have a long intrinsic setting time. The preparation technique has a significant effect on the powder morphology, specific surface area, stoichiometry, and crystallinity.

CHAPTER 3

BIOCOMPATIBILITY AND CYTOTOXICITY TESTING

3.1. Biocompatibility

Biocompatibility is broadly identified as the ability of a material to function in a specific application in the presence of an appropriate host response (Anderson, 2001). Biocompatibility may generally be regarded as the ability of a material to interact with living cells/tissues or a living system by not being toxic, injurious or without causing immunological reactions while performing or functioning appropriately (Hauman and Love, 2003). Toxicity refers to the potential harm that may be caused by a material when a biomaterial, prosthesis, or medical device is placed in the body results in injury to tissues or organs. It is this injury and the subsequent perturbation of homeostatic mechanisms that lead to the cellular cascades of wound healing. The response to injury is dependent on multiple factors including the extent of injury, the loss of membrane structures, blood-material interactions, provisional matrix formation, the extent or degree of cellular necrosis, and the extent of the inflammatory response. These events, in turn, may affect the extent or degree of granulation tissue formation, foreign body reaction, and fibrosis or fibrous capsule development. The host reactions are considered to be tissue, organ, and species dependent. In addition, it is important to recognize that these reactions occur very early, i.e. within 2 to 3 weeks of the time of implantation (Anderson, 2001).

Biocompatibility testing necessitated the development of methods to standardize biological tests for biomaterials, to find an effective and safe testing protocol that is more reliable for comparing results from different studies.

The determination of the biocompatibility of materials and implant devices involves detailed characterization of the material (e.g. bulk and surface chemical composition, density, porosity, and mechanical, electrical, and degradation properties) and extensive testing, first at the protein/ cell/ tissue or *in-vitro* level, and then *in-vivo* animal models and ultimately in human clinical trials (Williams, 2008).

Biocompatibility is measured with 3 types of biological tests: *in vitro* tests, animal experiments and clinical tests. *In vitro* tests are used to screen materials, their

components, and or leachable/ soluble/ degradation products for cytotoxic, genotoxic, immunological, and hemoliytic effects. Animal models are used to evaluate material-host tissue interactions and to predict how the device or prototype may perform in humans. Ultimately, the safety and effectiveness of the device must be evaluated in humans prior to widespread use by pysicians and their patients. At each stage, biocompatibility testing results must be correlated with materials properties and manufacturing, sterilization, packaging, storage and other handling procedures that also may influence test outcomes.

The design and use of biocompatibility testing protocols are provided by a variety of professionel and regulatory organizations including ASTM (American Society for Testing and Materials), ISO (International Standarts Organization, ISO standart 10993, ‘Biological Evaluation of Medical Devices’), ADA (American Dental Association), NIH (National Institutes of Health) and FDA (Food and Drug Administration). The use and documentation of biocompatibility tests are required by law in the United States and other countries.

The cytotoxicity of CPCs has been proven by extensive reported research. Guo et al. (2009) produced Ca-deficient hydroxyapatite (CDHA) porous scaffolds from calcium phosphate cement (CPC). TTCP and DCPA were mixed with distilled water and sodium chloride (NaCl) particles were used as porogen. HA scaffolds were also prepared using the same method as the control. The proliferation and differentiation of mesenchymal stem cells (MSCs) into osteoblastic phenotype on the scaffolds were determined using MTT assay, alkaline phosphatase activity and scanning electron microscopy. It was found that, CDHA and HA scaffolds have no negative effects on the viability and proliferation of MSCs. These two scaffolds both had good biocompatibilities.

Ishikawa et al. (2002) studied the effects of ZnTCP added to apatite cement (AC) with respect to its setting reaction and proliferation of human osteoblastic cells. Zn doped β-tricalcium phosphate (ZnTCP) powder was mixed into an equimolar mixture of TTCP and DCPA up to 10 wt% and was used as the powder phase of apatite cement (AC). Distilled water or 0.2mol/L neutral sodium hydrogen phosphate were used as cement liquid. Clonal human osteoblast cells were used in the MTT assay. The results showed that the proliferation of osteoblastic cells were quick on AC (5% ZnTCP) compared to AC (0% ZnTCP) and AC (10% ZnTCP). It was found that AC (10%) or a Zn content of 0.62% had toxic effect on the human osteoblastic cells.

Dagang et al. (2006) investigated the physicochemical properties and cytotoxicity of calcium phosphate cement (CPC) prepared by mixing TTCP and DCPA powders by using different concentrations of physiological saline or phosphate acid. MTT test for cytotoxicity was performed by adding different dilutions of calcium phosphate cement's (CPC) extract to an L929 cell culture. They reported that all extracts exhibited no or low cytotoxicity. CPC preperad by saline had higher relative growth rate (RGR) than CPC prepared by phosphate acid.

Lu et al. (2011) fabricated magnesium doped apatite cement (Md-AC) by adding the mixed powders of magnesium oxide and calcium dihydrogen phosphate (MO–CDP) into hydroxyapatite cement (HAC) prepared from tetracalcium phosphate (TTCP) and dicalcium phosphate anhydrous (DCPA) in an equal molar ratio. Water was used as the liquid component of the cement. They applied MTT test, morphological examination and alkaline phophatase activity on MG63 (Human osteosarcoma cell). It was found that Md-AC cement promote cell proliferation compared to HA cement. Md-AC was biocompatible with no obvious negative effects on cell growth.

3.2. Cell Culture

Cell culture refers to the removal of cells from an animal or plant and their subsequent growth in a favorable artificial environment. The cells may be removed from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain that has already been established. Primary culture is the stage of the culture after the cells are separated from the tissue and proliferated under suitable conditions until they occupy all of the available substrate. At this stage, the cells have to be subcultured (i.e. passaged) by transferring them to a new vessel with fresh growth medium to provide more room for continued growth. After the first subculture, the primary culture is called as a cell line or subclone. Cell lines derived from primary cultures have a limited life span, and as they are passaged, cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population. If a subpopulation of a cell line is positively selected from the culture by cloning or someother method, this cell line becomes a cell strain. A cell strain often earns additional genetic changes subsequent to the initiation of the parent line.

Culture conditions vary widely for each cell type, but the artificial environment in which the cells are cultured invariably consists of a suitable vessel containing a substrate or medium that supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, hormones, and gases (O_2, CO_2), and regulates the physicochemical environment (pH, osmotic pressure, temperature).

L929 mouse fibroblast cell culture was used in this study because it is appropriated for the *in-vitro* cytotoxicity test according to ISO 10993-5.

3.3. Cytotoxicity Tests

The cytotoxicity term is used to define the cascade of molecular events that interfere with macromoleculer synthesis, causing unequivocal cellular, functional and structural damage (Murray et al. 2007).

General toxicity tests, aimed mainly at detection of the biological activity of test substances, can be carried out on many cell types (e.g. fibroblasts, HeLa and hepatoma cells). A number of parameters including vital staining, cytosolic enzyme release, cell growth and cloning efficiency are used as end-points to measure toxicity.

Cytotoxicity tests are used as initial evaluation tests for devices principally contacting bone; examples include orthopaedic pins, plates, replacement joints, bone prostheses, bone cements and intraosseous devices. These tests designate the lysis of cells (cell death), the inhibition of cell growth, and other effects on cells result in medical devices, materials and/or their extracts, with the use of cell culture techniques. General guidance for *in vitro* cytotoxicity testing is presented in ISO 10993-5. Sensitization, irritation, intracutaneous reactivity, systemic toxicity (acute toxicity), subacute and subchronic toxicity, genotoxicity, implantation, and haemocompatibility are considered for every type of medical device for initial biological response after cytotoxicity tests (ISO 10993-1:2003E). The initial evaluation tests for implant device and duration category are given in Table 3.1.

Table 3.1. Initial evaluation tests for consideration (Source: ISO 10993-1:2003E).

Initial evalation tests for consideration										
Medical device categorization by			**Biological effect**							
Nature of body contact		Contact duration **A—Limited** (> 24h) **B—Prolonged** (24h to 30 days) **C—Permanent** (>30 days)	Cytotoxicity	Sensitization	Irritation or intracutaneous reactivity	Systemic toxicity (acut)	Subacute and subchronic toxicity	Genotoxicity	Implantation	Haemocompa tibility
Category	**Contact**									
External Communicating devices	Blood path indirect	A	x	x	x	x				x
		B	x	x	x	x				x
		C	x	x		x	x	x		x
	Tissue/bone/ dentin	A	x	x	x					
		B	x	x	x	x	x	x	x	
		C	x	x	x	x	x	x	x	
	Circulation blood	A	x	x	x	x				x
		B	x	x	x	x	x	x	x	x
		C	x	x	x	x	x	x	x	x

In vitro cytotoxicity assays measure whether a test compound is toxic to cells in culture, usually by determining the number of viable cells remaining after a defined incubation period (Stoddart, 2011). *In vitro* tests of cell toxicity provide a rough assessment of the ability of cells relevant to a determined application to survive in the presence of specific materials. In general, two different protocols are observed, one providing a direct contact of the materials with the cells, and another evaluating the effect of the contact with leachable materials (diffusible components, degradation products, etc.), thus considering an indirect cell contact (Rodrigues et al. 2012).

Extracts of test devices and materials are tested by exposure to the cell culture system (e.g. L929 mouse fibroblast cell line). The presence of cytotoxic leachates is

indicated by the loss of cell viability. The direct contact assay involves the placement of the test material directly on the cell culture medium. In these tests, approximately one-half million to one million cells are present in each culture dish, and toxicity is verified after a period of exposure (typically 24–72 hours) of the cells to the extract or device. Cytotoxicity is evaluated by qualitative (by examining the cells microscopically) and quantitative means (by measuring cell death, inhibition of cell growth, cell proliferation or colony formation). Positive control materials and negative control materials are similarly tested along side to validate the test results. Indirect method and direct method are schematized in Figure 3.1.

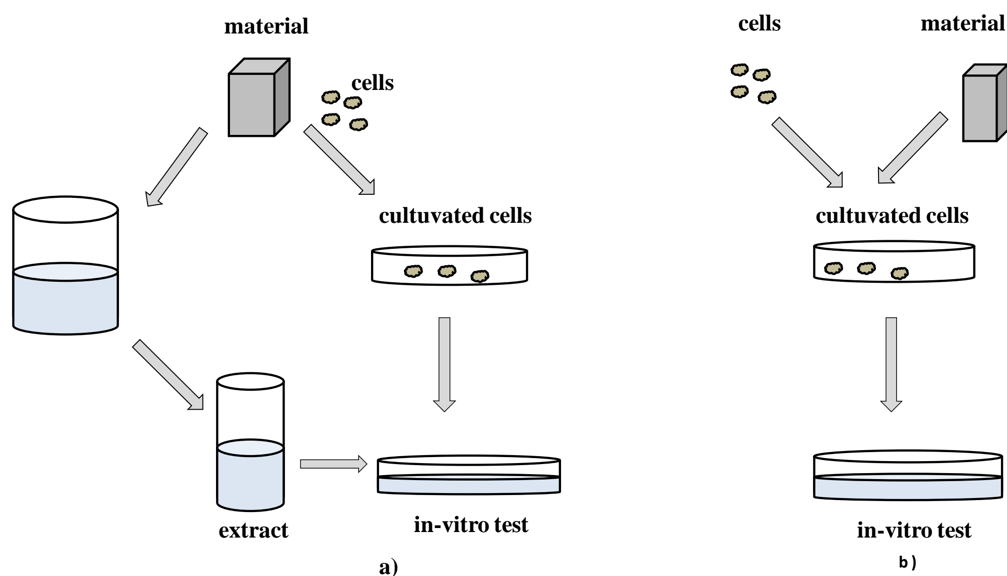
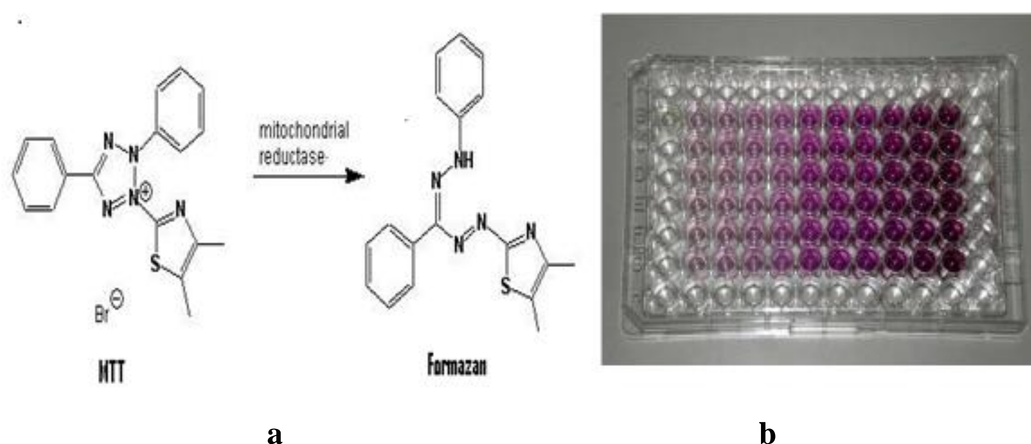


Figure 3.1. a) Indirect method: elution test, b) direct method: contact test.

3.4. MTT Cytotoxicity Test

The MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) cleavage assay is a sensitive, quantitative and reliable colorimetric assay that measure viability, proliferation and activation of cells or, when metabolic events lead to apoptosis or necrosis, a reduction in cell viability. The assay is based on the capacity of the cellular mitochondrial dehydrogenase enzyme in living cells to reduce the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2yl) -2,5-diphenyl tetrazolium bromide (MTT) into a dark blue/purple formazan product which is insoluble in water. After that

a solubilization solution (DMSO) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring with a spectrophometer. The amount of formazan produced is directly proportional to the cell number in a range of cells lines (Avila and Pugsley, 2011). For each treatment the percentage inhibition of growth is calculated. The MTT reduction by the mitochondrial dehydrogenase enzymes of living cells is schematized in Figure 3.2.



a **b**

Figure 3.2. a) The MTT reduction by the mitochondrial dehydrogenase enzymes of living cells b) A microtiter plate after an MTT assay. Increasing amounts of cells resulted in increased purple colouring.

According to, ISO 10993-5:2009 (E), the following formula is used to calculate cell viability:

$$Percent\ Cell\ Viability = \left[\frac{Number\ of\ live\ cells}{Number\ of\ total\ cells\ (live+dead)}\right] \times 100 \qquad (3.1)$$

If viability is reduced to < 70 % of the blank, material has a cytotoxic potential, on the other hand, reduction of cell viability by more than 30 % is considered a cytotoxic effect according to ISO 10993-5: 2009(E).

CHAPTER 4

MATERIALS AND METHODS

4.1.Materials

The formulas and sources of the chemicals used in powder synthesis and HA cement formulations are listed in Table 4.1.

L929 cell line derived from mouse fibroblast was used for the biocompatibility /cytotoxicity test. L929 cell line was kindly provided by Prof. Dr. Menemşe Gümüşderelioğlu from Hacettepe University.

Dulbecco's Modified Eagle Media (DMEM, Biological Industries), Fetal Bovine Serum (FBS, Sigma), trypsin, pen-strep solution, trypan blue, 3- (4,5-dimetiltriazol-2-il) -2,5 difeniltetrazoliumbromid (MTT),and dimethyl sulfoxide (DMSO) obtained from Sigma Chemical Company were used in L929 cell culture and MTT assay. Corning Star tissue culture plates and Greiner polypropylene tubes and disposable pipettes were used in this work.

Table 4.1 Chemicals used in HA cement formation.

Powders/Chemicals	**Chemical Formula**	**Formula Weight** g/mol	**Source**
Phosphoric Acid	H_3PO_4	97.995	MERCK
Calcium Carbonate	$CaCO_3$	100.0869	FLUKA
Calcium Nitrate Tetrahydrate	$Ca(NO_3)_2.4H_2O$	236.149	SIGMA-ALDRICH
Amonium Phosphate Monobasic	$(NH_4)H_2PO_4$	115	MERCK
Tetracalcium Phosphate	$Ca_4P_2O_9$	366	LAB MADE
Dicalcium Phosphate Dihydrate	$CaHPO_42H_2O$	172	LAB MADE
Amonium Phosphate Dibasic	$(NH_4)_2HPO_4$	132	MERCK
Hydroxyapatite	$Ca_{10}(PO_4)_6(OH)_2$	1004	SIGMA
Sodium Hydrogen Phosphate	Na_2HPO_4	141.96	MERCK
Sodium Phosphate Monobasic monohydrate	$NaH_2PO_4\,H_2O$	137.99	SIGMA-ALDRICH

4.2. Methods

4.2.1. Synthesis of TTCP and Brushite Powders

Tetracalcium phosphate powder (TTCP-1, from H_3PO_4 and $CaCO_3$) was prepared by heat treatment of calcium and phosphate precursor mixtures at 1350°C followed by quenching to room temperature in a dessicator. A predetermined amount of 85% phosphoric acid (14.73 mL of the acid equivalent to 0.22 mol of H_3PO_4) was added to130 mL ultrapure water in a beaker for the acidic solution preparation. The necessary amount of calcium carbonate (0.442 mol $CaCO_3$) was further added to this acidic solution under constant stirring. This mixture was ultrasonically treated for 10 minutes in a laboratory ultrasonic cleaner. The solvent (water) of the suspension was further removed by boiling for about 1 h on a hot plate under constant stirring. The dried solid mixture was further dried at 70°C in an oven for 2 days. The dried mixture was heat treated at 1350°C with a heating rate of 10°C/min with 5 hours hold at the soak temperature in a Carbolite RHF 1600 muffle furnace and quenched from 1250°C to room temperature after heat treatment cycle. The heat treated solid which was expected to be TTCP was placed into a desiccator at room temperature. The synthesized TTCP powder was further ground by an agate mortar to obtain a TTCP powder after cooling.

TTCP-2 was prepared by heat treatment of amonium phosphate monobasic ($(NH_4)H_2PO_4$) and calcium carbonate ($CaCO_3$) at 1350 °C followed by quenching to room temperature in a dessicator. 0.2 mol of $(NH_4)H_2PO_4$ was added to 130 mL ultrapure water in a beaker. The necessary amount of calcium carbonate (0.442 mol $CaCO_3$) was further added to this solution under constant stirring. This suspension was ultrasonically treated for 10 minutes and the suspension was dried in about 1 h on a hot plate under constant stirring. The dried solid mixture was further dried for 4 days in an oven at 80°C. The dried precursor mixture was heat treated at 1350°C (heating rate of 10°C/min) with 5 hours hold at the soak temperature in a Carbolite RHF 1600 muffle furnace and quenched from 1250°C to room temperature after heat treatment cycle. The heat treated solid mass was placed into a desiccator at room temperature and was further ground by an agate mortar after cooling.

Dicalcium phosphate dihydrate (DCPD or Brushite) was synthesized by a chemical precipitation method in aqueous solutions at room temperature by using

calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$) and amonium phosphate dibasic ($(NH_4)_2HPO_4$) salts by adjusting the Ca/P molar ratio to 1. Two solutions were used to form DCPD. The first solution was prepared by dissolving 0.4 mol Ca $(NO_3)_2.4H_2O$ in 800 ml deionized water and the pH of the solution was adjusted to 4 by the addition of 1:1 concentrated nitric acid (HNO_3)-H_2O solution at room temperature.

The second solution was prepared by dissolving 0.4 mol of $(NH_4)_2$ HPO_4 in 800 ml of deionized water, pH of the solution was adjusted to 4 by the addition of 1:1 concentrated nitric acid (HNO_3)-H_2O solution at room temperature. The first solution was then added into the second solution via a peristaltic pump and the precipitates were formed. The pH of the solution was kept at 4 by the addition of 1:1 diluted ammonia (NH_3OH)-H_2O solution at room temperature. The suspension was centrifuged for 10 minutes at 4000 rpm. Precipitate was further washed by the addition of 2 liters of deionized water with an adjusted pH of 4 and were centrifuged for 5 min. at 6000 rpm. Washing step was repeated 6 times with deionized water and conductivity of the supernatant was finally measured as 118. 7 µs/cm. The precipitate was further dried for 2 days at 37 °C. DCPD powders was ground by an agate mortar initially and wet ground in a planetary ball mill for 1.5 h afterwards.The particle size was measured by Malvern Zetasizer 3000HSA.

4.2.2. Hydroxyapatite Bone Cements Preparation

Hydroxyapatite bone cement specimens were prepared by mixing the solid and liquid components. The liquid component consisted of 0.2 and 0.3 M phosphate buffer solutions (PB). Two separate solutions were used for the preparation of PB. The first solution was prepared by dissolving 0.02 mol of NaH_2PO_4 in 100 mL of deionized water. The second solution (0.2 M Na_2HPO_4 solution) was prepared by dissoving 0.02 mol Na_2HPO_4 in 100 ml deionized water. 0.2 M phosphate buffer solution (PB) at pH 7.4 was prepared by mixing 40 ml of sodium dihydrogen phosphate solution(Solution 1) and 10 ml of sodium phosphate dibasic (Na_2HPO_4) solution (Solution 2). 0.3 M PB was prepared similarly with appropriate amounts of both solutions.

Hydroxyapatite (HA) cement pastes (with Ca/P of 1.67) were prepared by mixing tetracalcium phosphate powder (TTCP-1) with brushite powder (DCPD) and phosphate buffer solution (PB) as the cement liquid. HA cement pastes were further

placed in an injector using a spatula and packed tightly with the plunger for the formation of about 5 mm by 4 mm (diameter-height) cylindrical set cement samples (Komath et al. 2003). This sample dimension was chosen to be appropriate for placing in the 24-well plates (15.6 mm well diameter).

Setting time of HA cements was determined by using a home-made Vicat Needle Apparatus. Eighteen different HA cements were prepared by changing solid/liquid (S/L) ratio, seed (HA) content and PB solution molarity. A picture of a commercial calcium phosphate cement kit is given in Figure 4.1. Photographs of the cement samples prepared in this work are given in Figure 4.2. The cement formulations investigated are also tabulated in Table 4.2.

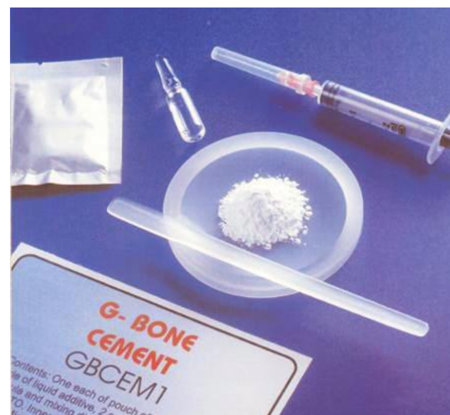


Figure 4.1. The picture of commercial calcium phosphate cement kit (Source: Dorozhkin, 2013).

Figure 4.2. The picture of hydroxyapatite cement samples.

Table 4.2. Formulations of HA cements investigated.

Sample Code	**Phosphate Buffer Solution (M)**	**Solid(g)/Liquid(mL) Ratio (TTCP+ DCPD/PB)**	**Seed Concentration % HA**
HA-1	0.2	3.2	-
HA- 2	0.2	3.2	3
HA -3	0.2	3.2	1.5
HA -4	0.2	2.7	-
HA -5	0.2	2.7	3
HA -6	0.2	2.7	1.5
HA -7	0.2	2.4	-
HA -8	0.2	2.4	3
HA -9	0.2	2.4	1.5
HA -10	0.3	3.2	-
HA -11	0.3	3.2	3
HA-12	0.3	3.2	1.5
HA -13	0.3	2.7	-
HA -14	0.3	2.7	3
HA -15	0.3	2.7	1.5
HA -16	0.3	2.4	-
HA -17	0.3	2.4	3
HA -18	0.3	2.4	1.5

4.2.3. Characterization of Calcium Phosphate Powders and HA Cements

The phase compositions of TCCP-1, TCCP-2, brushite powders and the HA cements (CPCs) were determined by Philips X'pert Pro X- ray diffractometer. The microstructure and morphology of synthesized powders and HA cement samples were investigated by using a Philips XL-30S FEG scanning electron microscope.

4.2.4. Setting Time Measurements

Setting of calcium orthophosphate formulations is a continuous process that always starts with dissolution of the initial compounds in an aqueous system. This process supplies calcium and orthophosphate ions to the solution where they chemically interact and precipitate in the form of either the end-products or precursor phases which causes the cement setting.

Particle size, S/L ratio, temperature and initial presence of HA powders as seeds in the solid phase influence the setting time. The setting time can be controlled by using additives in the liquid phase. CPCs must set slowly enough to provide sufficient time to a surgeon to perform implantation but fast enough to prevent delaying the operation. Good mechanical properties should be reached within minutes after initial setting (Dorozhkin. 2013). However setting times that exceed 30 minutes are clinically limited; therefore much work has been directed towards the use of additives as a means of reducing the setting times of these cements.

Setting time of HA cements was determined by using a home-made Vicat Needle Apparatus with weight and sizes described in ‘ASTM C 187-04 Standart test method for normal consistency of hydraulic cement’. In this method predetermined amounts of powder and PB were mixed with a spatula. Cement paste was filled into an injector by using a spatula. The cement pastes were considered set when 300 g weight loaded onto a Vicat Needle apparatus does not form a visible circular indentation on its surface with a tip diameter of 1 mm.

4.3. Cell Culture Experiments

4.3 1.Preparation of Cell Strains

The mouse fibroblast cell line L929 was obtained from Hacettepe University. L929 and Dulbecco’s Modified Eagle’s Medium (DMEM), which consisted of 10% fetal bovine serum (FBS), along with antibiotics were placed in a 75ml flask. They were then incubated at 37°C under a humid atmosphere of 5% CO_2 for 24 h. The culture medium was discarded from the flask and the cells were washed with 10 mL of

phosphate buffer saline (PBS). The cell layer was detached from the flask by the addition of 3 mL trypsin solution. After the addition of 9 mL DMEM-FBS to this cell solution, the suspension was centrifugated to recover the cells. The supernatant after centrifugation was discarded and 2 mL DMEM was added to obtain cell suspension. Cells from the sixth passage were used in these experiments. Cultivation of L929 cells in flasks containing DMEM- FBS are shown in Figure 4.3**.** The aliquots of this suspension were counted in a Neubauer hemocytometer. The suspension was adjusted to 1 x 10^4 cells/mL and inoculated into a petri plate (96 wells).

Figure 4.3. Cultivation of L929 Cells in flasks containing DMEM- FBS.

4.3.2. MTT Cell Viability Test for Powders

Cytotoxicity testing was performed using the MTT (3-(4.5-dimethylthiazol-2-yl)2.5-diphenyl tetrazolium bromide) cleavage assay to determine the number of viable cells in culture.

The cell culture cytotoxicity studies of brushite and TTCP powders were performed by indirect method (according ISO 10993-5: 1999-E) using mouse fibroblast cells. Determination of cytotoxicity of the synthesized powders was conducted by using a modified version of the protocol applied by Dagang and colleagues (Dagang et al. 2006).

The test samples (about 1 gram of TTCP-1, TTCP-2 and brushite powders) were sterilized with ethylene oxide gas for 24h. The test samples were placed in 50mL conical centrifuge tubes along with 10 mL of DMEM- FBS culture medium containing 10 vol % of fetal bovine serum. All preparations were carried out in a clean bench under sterilization. The tubes were then incubated in a humid atmosphere of 5% CO_2 at 37°C

for 24h. The samples were centrifuged for 10 minutes at 4000 rpm then filtered through a 0.22 μm milipore membrane. Samples containing 100, 50, 25 and 10 vol % pure extract were prepared by using the supernatant and DMEM.

L929 cells were plated in a 96-well plate at a density of 1.0×10^5 cells per well. After cell inoculation and adhesion in a petri plate (96 wells) for 24 hours the medium was removed and 100 μL of each (100, 50, 25 and 10 vol %) powder extract and control was added to the well plate inoculating L929 cells. Triplicate of the tested extract samples was conducted for the determination of the mean cell viabilities. The plate was incubated in a humid incubator with 5% CO_2 at 37°C for 24, 48, and 72 hours. 100μL of 1:10 MTT-DMEM stock solution (5 mg MTT / mL) was added to each well after preset incubation periods. Incubation was continued for 4 h under similar conditions after the MTT additions. Plates were centrifuged at 1800 rpm for 10 minutes at room temperature in a plate rotor (Hettich 30 RF Centrifuge) to avoid accidental removal of formazan crystals. Supernatant removing by tapping on a paper towel was followed by dimethyl sulfoxide (100 μL) addition and mixing on an orbital shaker at 110 rpm for 10 min. The absorbance of the samples were measured at 540 nm by using an automated plate reader (Organon Teknica Reader 230S Version 1.22). The L929 cell culture which was not exposed to any powder extract was used as negative control.

Optical microscopic observation of the control and the powder extract treated cell cultures were conducted in order to witness cell viability. Figure 4.4 illustrates the appearance after an MTT assay of powder extracts incubated for 48h.

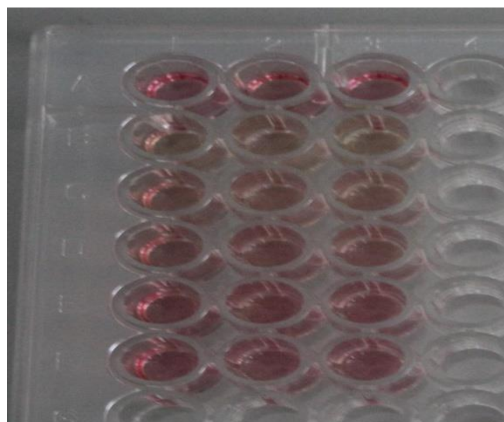


Figure 4.4. A petri plate after an MTT assay of powder extracts incubated for 48h. Increasing amounts of cells resulted in increased purple coloring.

4.3.3. MTT Cell Viability Test for Hydroxyapatite Cements

Eighteen different CPCs (HA-1 to HA-18) were sterilized with ethylene oxide gas initially. The cell culture cytotoxicity studies of HA cements were performed in a direct method (according ISO 10993-5: 1999-E; 2005) by using L929 fibroblast cells.

The L929 cells were seeded into a petri plate (24 well) for 24 hours at 37°C with 5% CO_2 humidified incubator. The HA cement samples were afterwards added to these plates. Inoculation of the L929 cells with the cement samples were conducted for 24, 48 and 72 hours at 37°C with 5% CO_2 humidified incubator. A picture of HA cement samples in a well plate containing L929 cell culture medium is shown in Figure 4.5. Triplicate of the tested HA cement samples was conducted for the determination of the mean cell viabilities. A similar procedure described for extracts was applied for the determination of the cell viabilities.

Figure 4.5. HA cement samples in a well plate containing L929 cell culture medium.

CHAPTER 5

RESULTS AND DISCUSSION

5.1.Characterization of Powders and Hydroxyapatite Cements

5.1.1. XRD Analysis

The phase compositions of brushite, TTCP-1, TTCP-2 powders and HA cement samples were characterized by XRD. XRD pattern given in Figure 5.1 shows the presence of the characteristic peaks of synthesized brushite. The detected phase is in good aggrement with the JCPDS card number 72-0713 for brushite. The major characteristic planes of brushite can be identified at 11.60. 20.75 and 29.21°as shown in Figure 5.1.

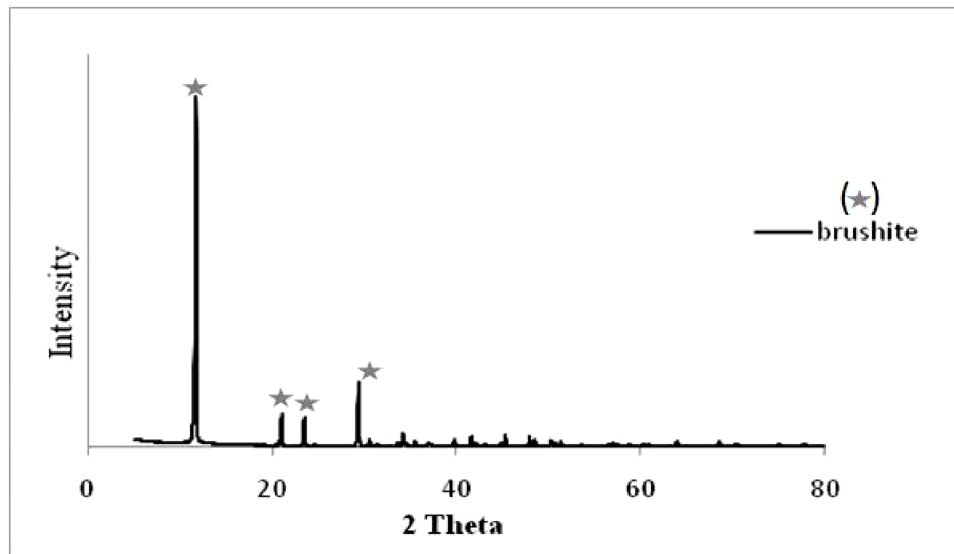


Figure 5.1. XRD pattern of brushite powder (* Brushite peak positions).

XRD patterns of the tetracalcium phosphate powders indicated the presence of the characteristic peaks of TTCP powders as shown in Figure 5.2. Major peaks of TTCP powders were identified at 25.60^o, 29.88^o and 32.35^o for TTCP-1 and 25.47^o, 29.81^o and 32.35^o for TTCP-2. The detected phase is consistent with the JCPDS card number- 70-1379 for TTCP.

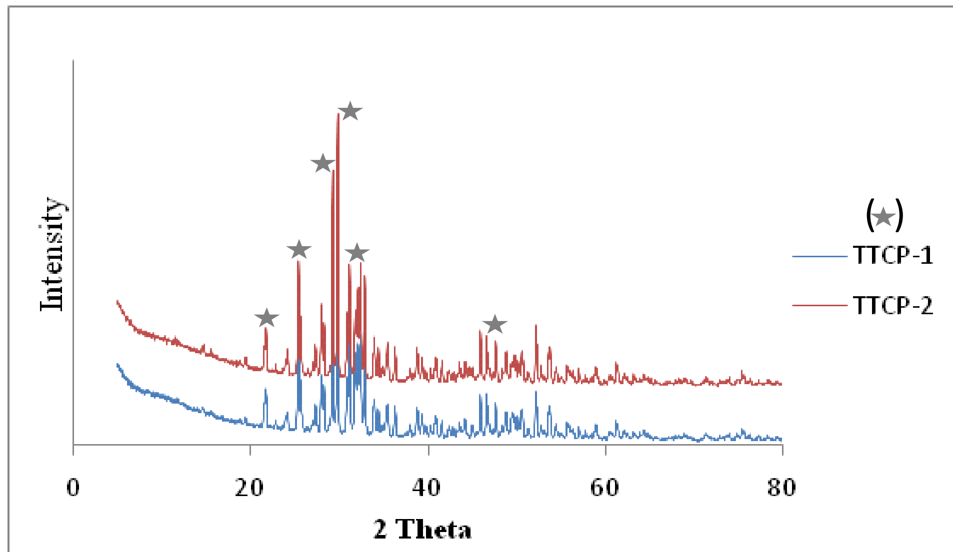


Figure 5.2. XRD patterns of TTCP-1 and TTCP-2 powders (★ TTCP peak positions).

The phase compositions of some calcium phosphate cements were determined by X-ray diffraction. The XRD patterns of the cements and the standard peaks of hydroxyapatite are shown in Figures 5.3, 5.4, 5.5, 5.6 and 5.7. The XRD pattern of HA exhibits many peaks in the 20°-60° 2θ range but the major peaks of HA were observed in the 25°-33° 2θ range. These determined patterns of the cement phases are consistent with the JCPDS card number 72-0713 for HA. The major phase was found to be hydroxyapatite as was expected. It is evident that the brushite and TTCP powders both dissolved and hydroxyapatite phase was precipitated. The XRD patterns of the cements proved that they are formed from a phase which is similar to the inorganic major phase of bone. It was observed that fast setting HA cements had smaller crystallite sizes and had lower crystallinity than slow setting HA cements. The addition of commercial hydroxyapatite as a crystallization seed also increased the crystallinity of HA cement which was concluded from the XRD patterns given in Figure 5.4.

The addition of various compounds, inorganic or organic, to calcium phosphate cement pastes is known to affect the setting time and crystal growth, sometimes to a great extent. Some of them were reported to inhibit the crystal growth (Bercier et al. 2010). The cement microstructures of HA-3, HA-12 and HA-18 obtained with seeds had lower crystallinity when compared with HA-4, HA-10 and HA-16 cement structures without seeds as shown in Figures 5.3, 5.5 and 5.7 respectively. The presence of seeds resulted in smaller cement crystallites during the cement formation process.

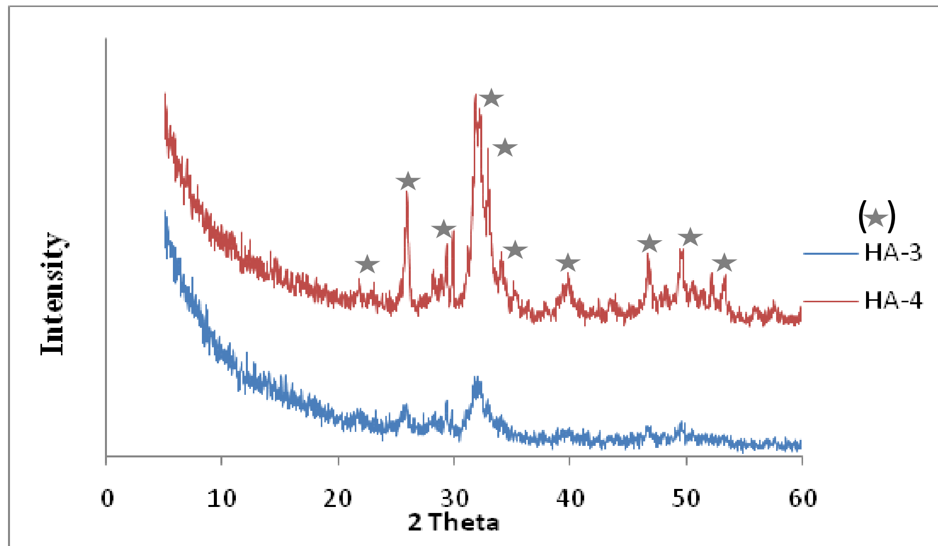


Figure 5.3. XRD graphs of HA-3 and HA-4 cements (⁎ HA peak positions).

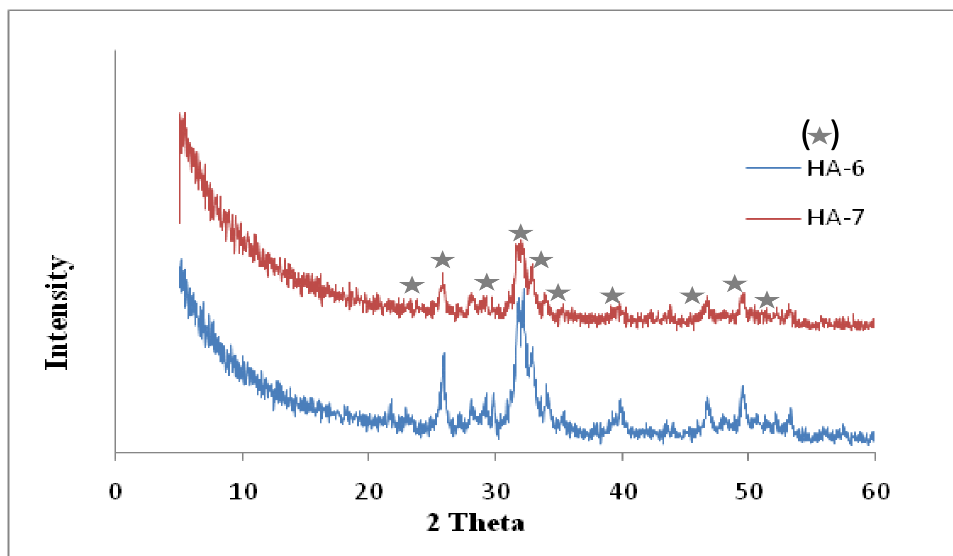


Figure 5.4. XRD graphs of HA-6 and HA-7 cements.

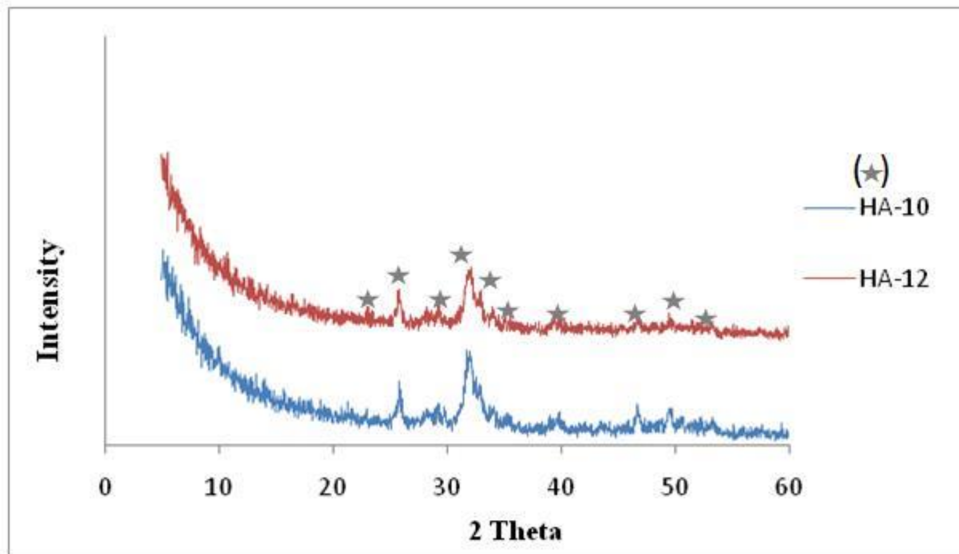


Figure 5.5. XRD graphs of HA-10 and HA-12 cements.

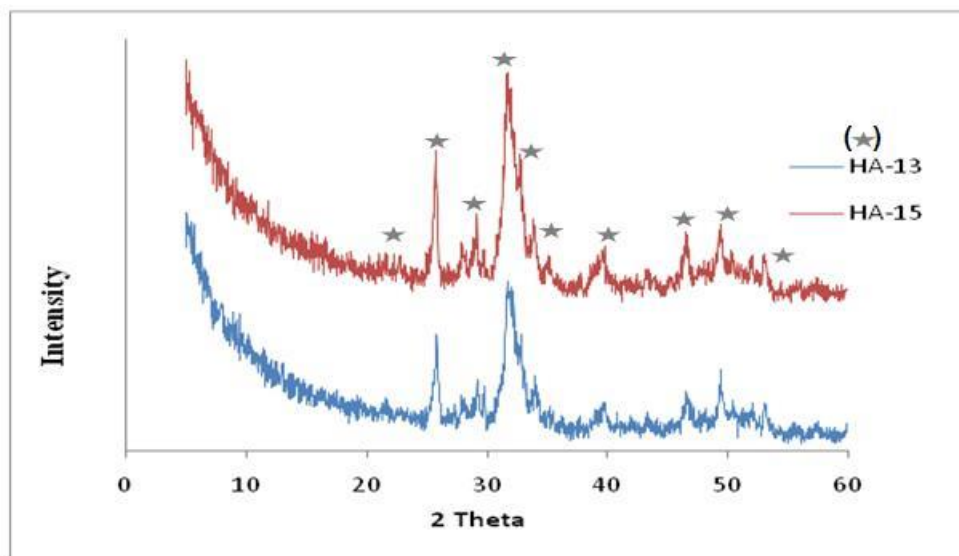


Figure 5.6. XRD graphs of HA-13 and HA-15 cements.

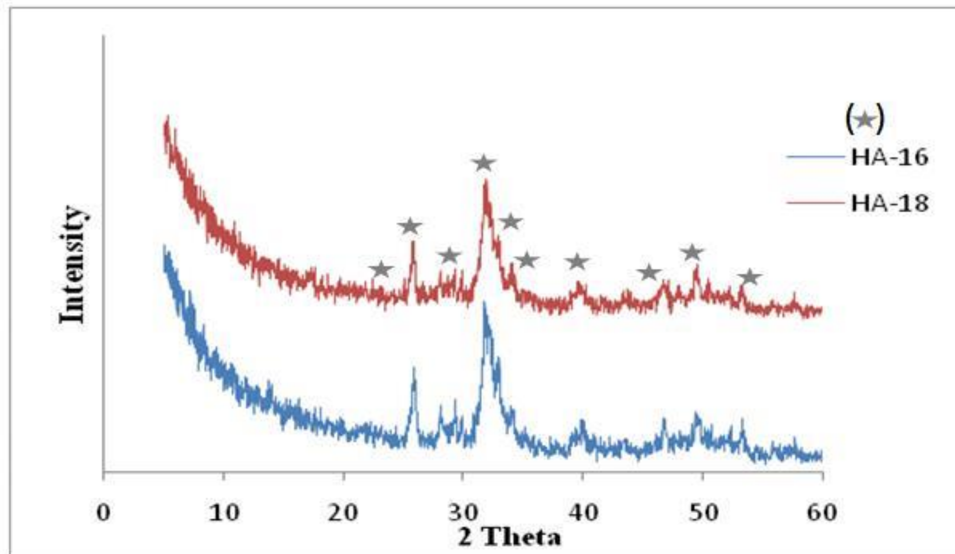


Figure 5.7. XRD graphs of HA-16 and HA-18 cements.

5.1.2.SEM Analysis

Scanning electron microscopy (SEM) images of the brushite powder are shown in Fig 5.8. The images illustrate the typical microstructure of thick platelets of the brushite crystals.

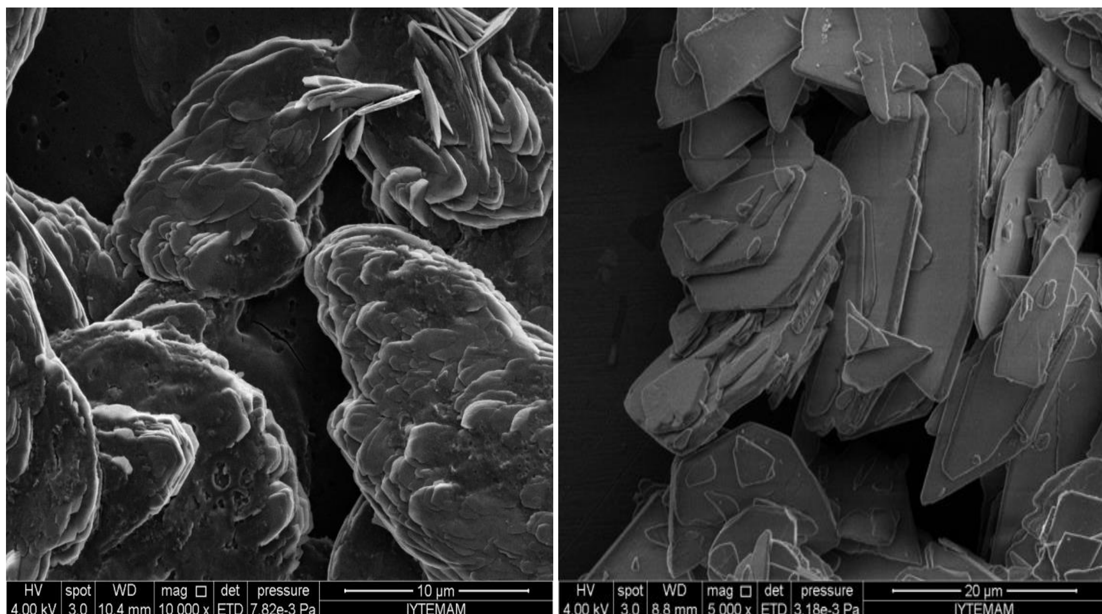


Figure 5.8. SEM image of synthesized brushite powder.

TTCP powders exhibit characteristic high temperature synthesized particle morphology with an aglomerated structure. Relatively large 10-20 μm particles with rounded surfaces of TTCP particle morphology can be clearly sen in Figure 5.9.

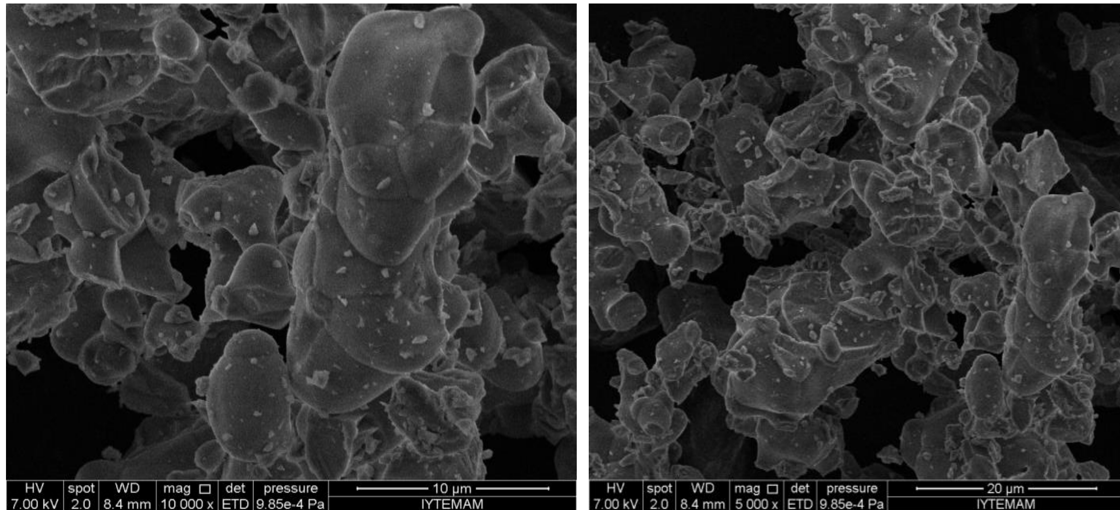


Figure 5.9. SEM image of synthesized TTCP powders.

SEM image of selected HA cements prepared with 0.2 M and 0.3 M phosphate buffer solution are given in Figures 5.10 and 5.11. As can be seen from the morphologies of the particles, there is a distribution of small particles and large agglomerates of HA. It is not possible to clearly observe hydroxyapatite crystals in SEM images of HA-2 (Fig. 5.10a.). HA crystals were found in the SEM image of HA-7. However brushite structure were not observed (Fig.5.10b.). In almost every part of the samples of HA-11-14-15-16 flower-shaped HA crystals were observed (Fig. 5.10c. 5.10d. 5.10e. 5.10f).

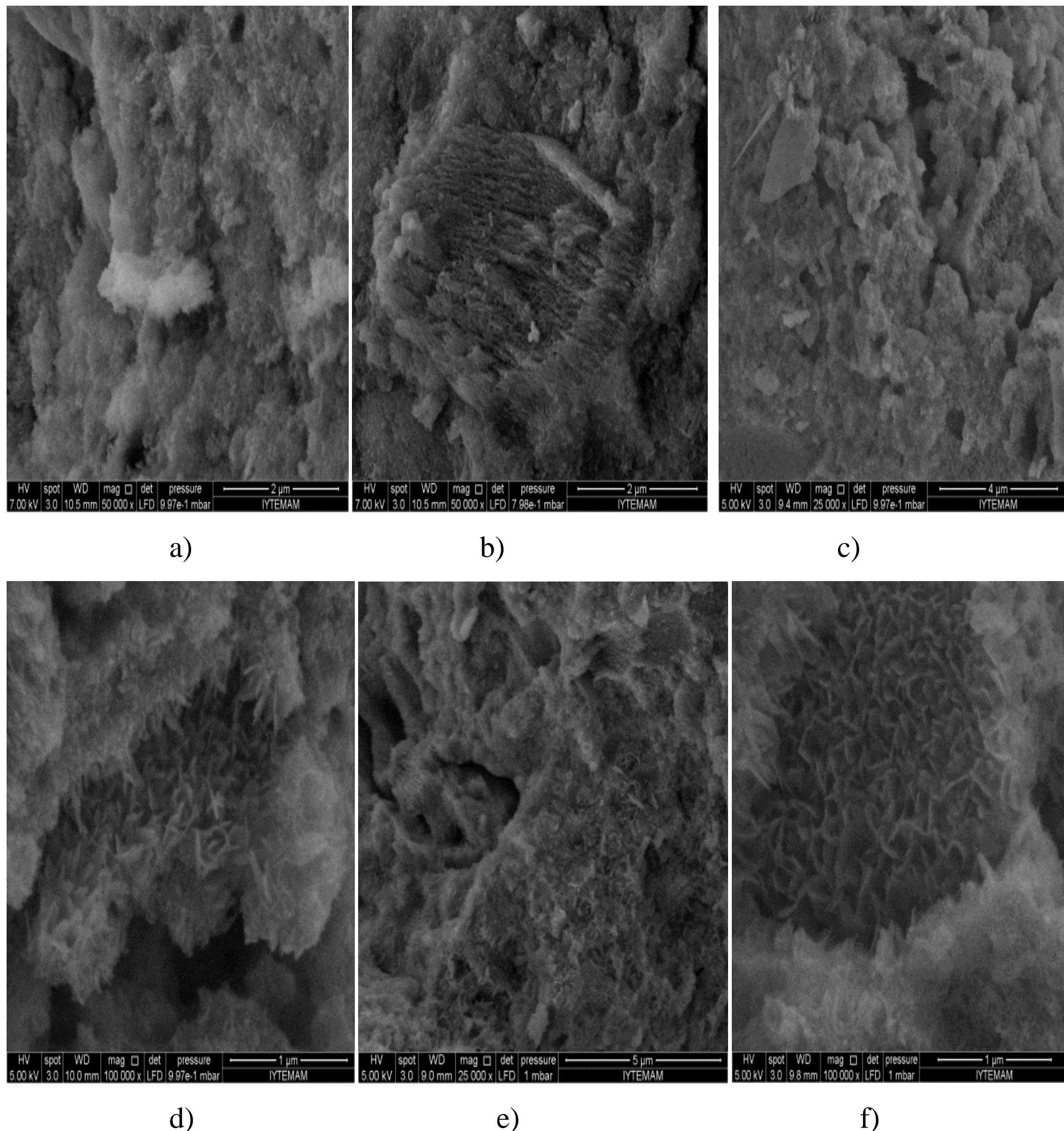
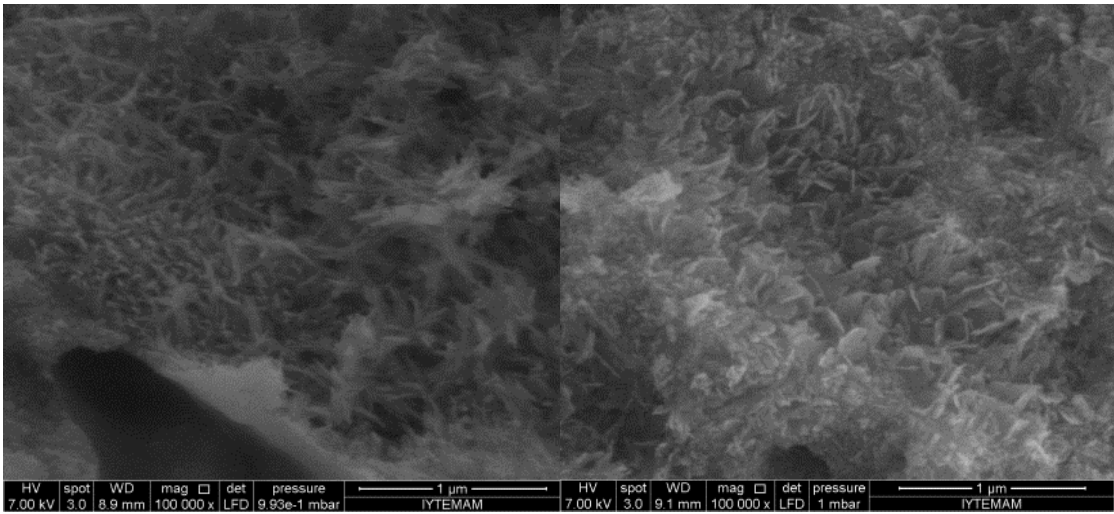
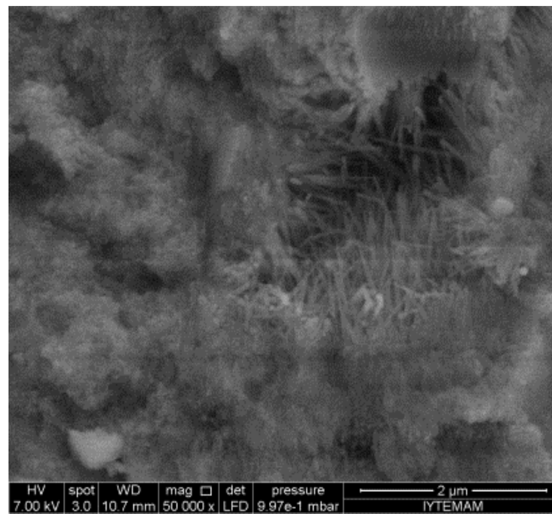


Figure 5.10. SEM image of prepared a) HA-2, b) HA-7, c) HA-11, d) HA-14 e) HA- 15, f) HA-16 cements.

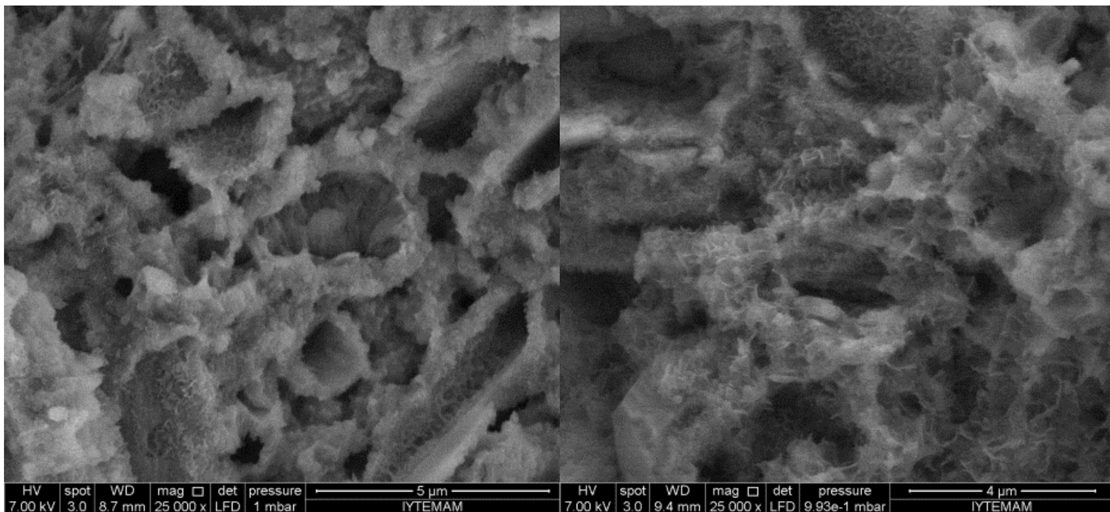
The microstructure of needle-like crystals of the hydroxyapatitic calcium phosphate cement formed by the entanglement of the precipitated crystals is seen in Figure 5.11. A mixture of needle-like HA crystals in almost everywhere were observed Fig. 5.11a, 5.11b, 5.11d and 5.11e. There was larger rod-shaped acicular crystals in some areas in the fracture surface of HA-12 (Fig. 5.11c).



a) b)



c)



d) e)

Figure 5.11. SEM image of selected HA cements. a) HA-3, b) HA-8, c) HA- 12, d) HA-13, e) HA-17.

5.1.3. Setting Time Measurements

The setting times of the HA cements were measured using cements prepared with 0.2 M and 0.3 M phosphate buffer (PB) solution at pH 7.2. Results indicated that the setting times of the HA cements were in the range of 3.5-24 minutes. HA-2 prepared with 0.2 M PB, the highest S/L ratio and 3% seed concentration showed the shortest setting time (3.5 min.) while HA-16 prepared with 0.3 M PBS, the lowest S/L ratio and 0 % seed concentration had the longest setting time. Except for the HA-11 cement, 3 % seed concentration was found to be more effective on decreasing the setting time compared to other seed concentrations (1.5 % and 0 % seed). According to the results of our work it can be said that the dominant parameter on the level of the setting time of the cements is the S/L ratio. The setting time increases with increasing solid/liquid ratio. The setting times of all HA cements are shown in Figure 5.12 and Table 5.1. Setting time plots of HA cements prepared using 0.2 M and 0.3 M PB solutions as a function of S/L ratio and seed concentration are also given in Figure 5.13 and Figure 5.14. 3% seed concentration and 3.2 S/L ratio were found to be most effective parameter especially, on the shortening of the setting time of HA cements as shown in Figure 5.13 and Figure 5.14.

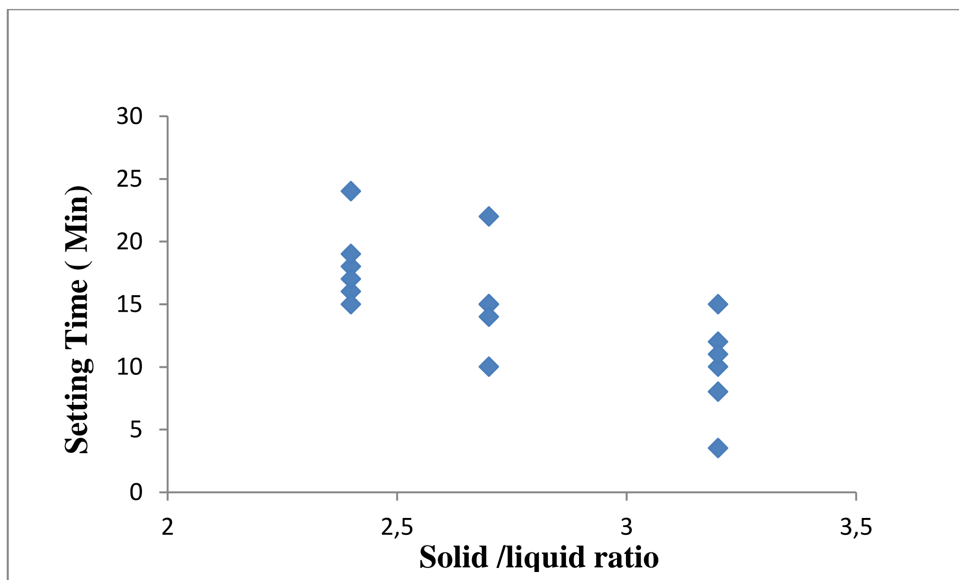


Figure 5.12. The setting times of all HA cements.

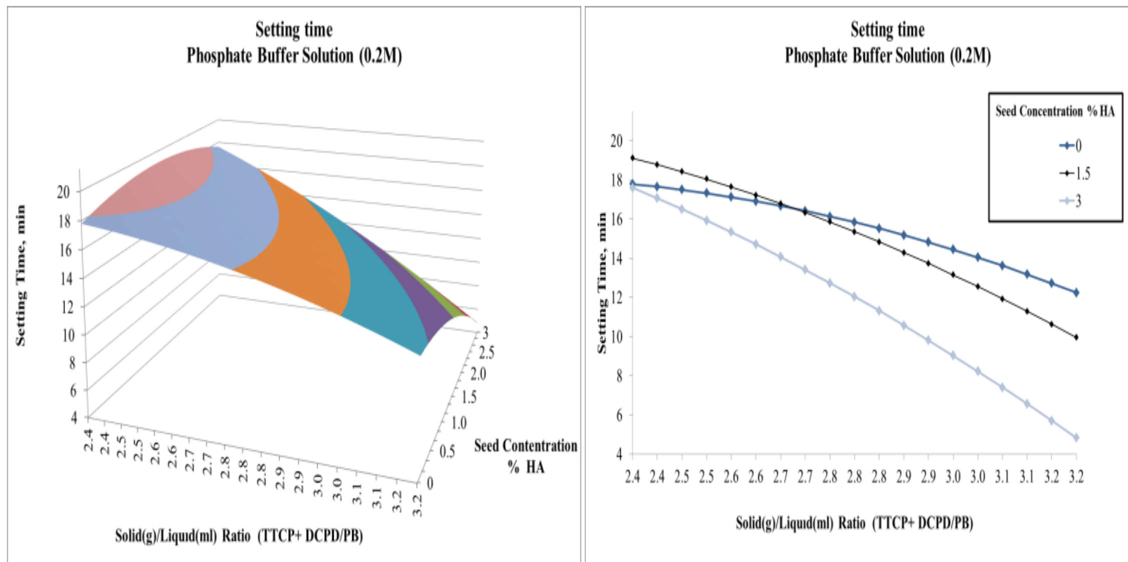


Figure 5.13. Setting time plots of HA cements prepared using 0.2 M PB solution as a function of S/L ratio and seed concentration.

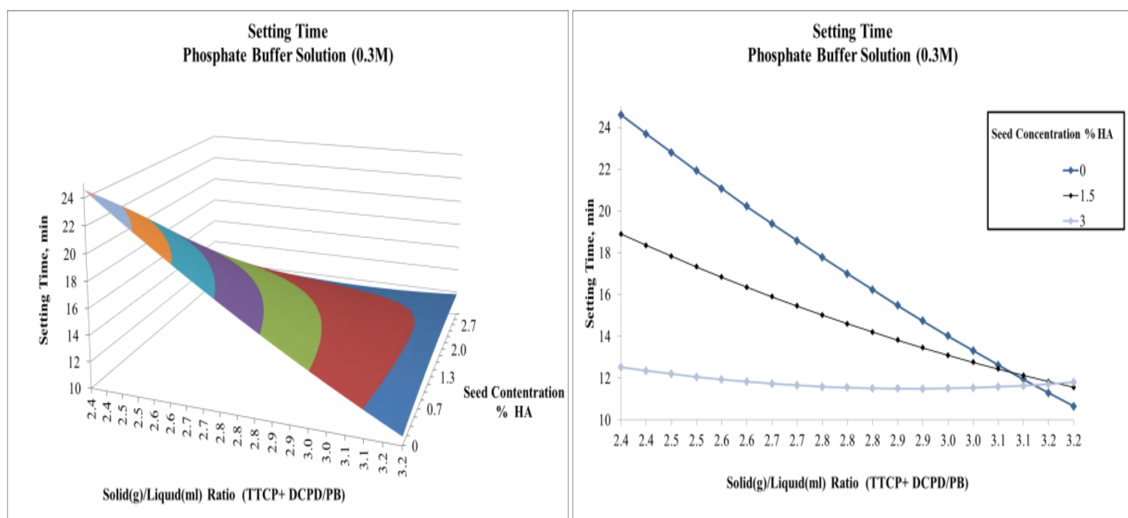


Figure 5.14. Setting time plots of HA cements prepared using 0.3 M PB solution as a function of S/L ratio and seed concentration.

Table 5.1. The setting times of the HA cements

Setting Time			
0.2 M PB	**minute**	**0.3 M PB**	**minute**
HA-1	12	HA-10	8
HA-2	3.5	HA-11	11
HA-3	10	HA-12	15
HA-4	15	HA-13	22
HA-5	15	HA-14	10
HA-6	15	HA-15	14
HA-7	18	HA-16	24
HA-8	16	HA-17	15
HA-9	19	HA-18	17

5.2. Cytotoxicity Results of Hydroxyapatite and Powder Samples

The cytotoxicity of the brushite, TTCP-1 and TTCP-2 powders was assessed by culturing L929 cells with extract fluids of powders for 24, 48 and 72h. The media prepared by using 100, 50, 25 and 10 vol % pure extract of the powders were used to construct the dose response curve. Culture medium with the cells without powder extract served as a negative control in all viability experiments. All experiments were repeated three times in order to obtain reliable results and on powder toxicity on cell viability after 24, 48 or 72 hour exposure to the extracts. Data given in this thesis represents the average of three different experiments.

Brushite caused sharp decreases in cell viability at the end of 24, 48 and 72 hours at all extract concentrations as shown in Figure 5.15. Cell viability levels were determined as 83 % (10 % extract) and 39 % (100 % extract) at the end of 24 h. Extract concentrations of 50 and 25 % however exhibited 52 % and 56% cell viability, respectively. Cell viability was found to be higher than 70 % at all extract concentrations as shown in Fig.5.15, Table 5.2, 5.3 and 5.4 except 100 % extract concentration which exhibited 60 and 44 % cell viability after 48 and 72 hours. These results imply that brushite powder has toxic effect on the cell viability at 50 % and 100 % extract concentrations compared to control.

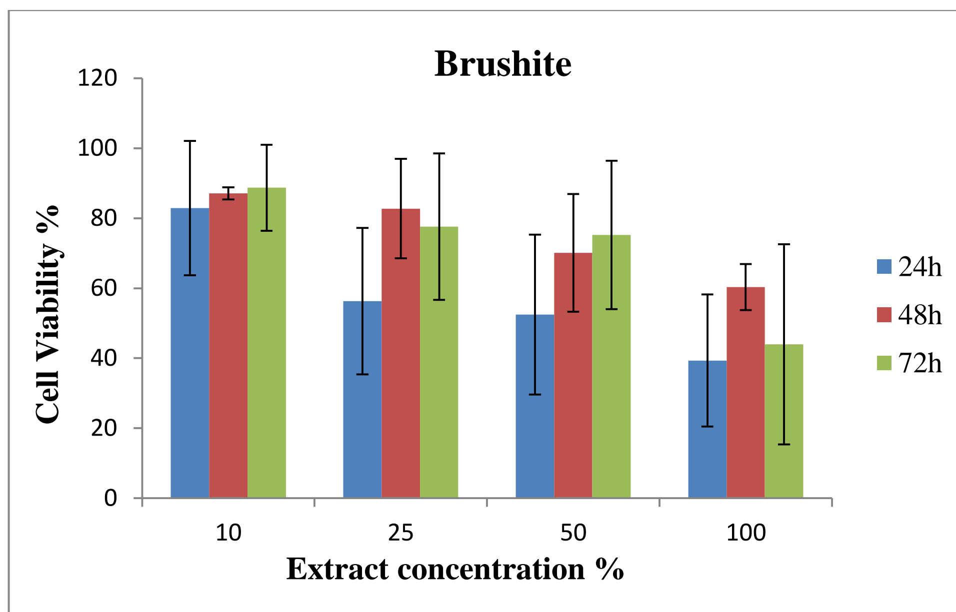


Figure 5.15. Cytotoxicity results of the brushite powder cultured for 24, 48 and 72 hours.

TTCP-1 did not show toxic effects on the cell viability at concentrations from 10 % up to 100 % as shown in Figure 5.16. According to cytotoxicity results at the end of 24 hours, cell viability stayed almost at the same level (around 80 %) at all extract concentrations. Similarly, cell viability values were found to be equal or above 83 % and 87 % after 48h and 72h, respectively.

After 24 hours the cell viability values obtained using TTCP powder extracts indicated that TTCP powder is not cytotoxic with cell viability values of 74 % and over (Figures 5.16 and 5.17). These results imply that TTCP-1 and TTCP-2 has no toxic effects on cell viability.

The cytotoxicity test results for brushite, TTCP-1 and TTCP-2 powders at the end of 24, 48and 72 hours are also tabulated in Tables 5.2, 5.3 and 5.4. Cell viability of brushite powder decreased upon increasing the extract concentrations in dilutions in contrast to the TTCP powder extracts.

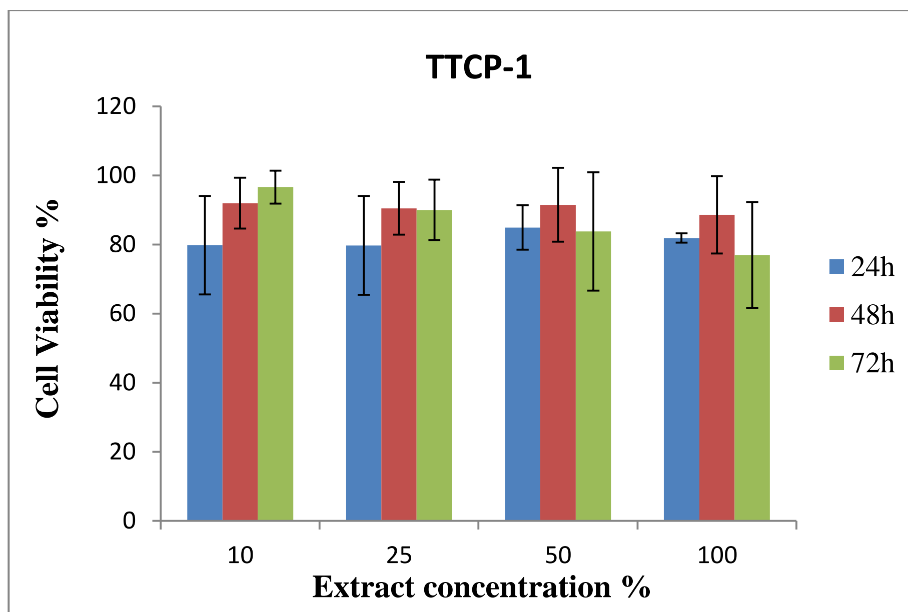


Figure 5.16. Cytotoxicity results of the TTCP-1 powder extract cultured for 24, 48 and 72 hours.

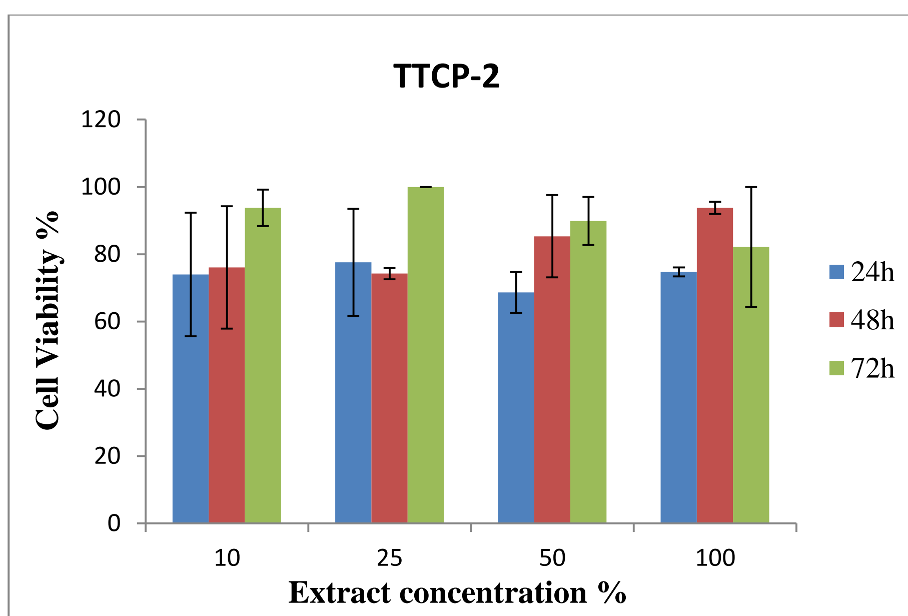


Figure 5.17. Cytotoxicity results of the TTCP-2 powder extract cultured for 24, 48 and 72 hours.

The cell viabilities of HA-1, HA-2 and HA-3 cements prepared by using 0.2M PB solution and 3.2 gr/mL S/L ratio after 24 hours were determined to be higher than 74 %. Cell viability values were 76 % and over after 48h and 78 % and over after 72h. Cell viability values therefore indicated that these cements do not exhibit a significant difference on the cell viability and have no toxic effects on the L929 mouse fibroblast

cells. The cytotoxicity results of the HA-1, HA-2 and HA-3 cements for 24, 48 and 72 hours are also shown in Figures 5.18 and Table 5.5.

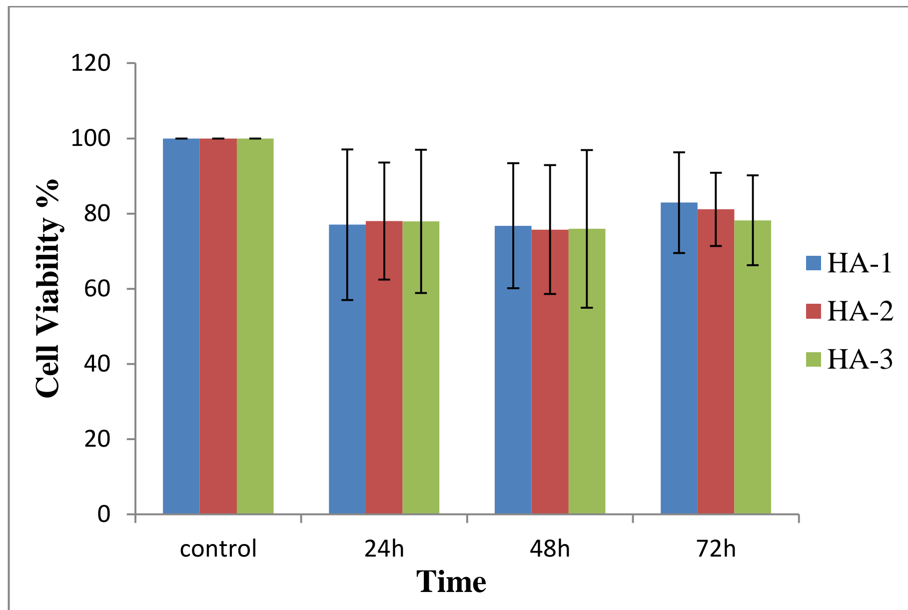


Figure 5.18. Cytotoxicity results of the HA-1, HA-2 and HA-3 cements cultured for 24, 48 and 72 hours.

The cements HA-4, HA-5 and HA-6 prepared using 0.2M PB with a 2.7 gr/ml solid/liquid ratio also didn’t had any toxic effects on the cell viability compared to the control. The results obtained for 24 hours indicated cellular viabilities of 77 %, 75 % and 80 % for HA-4, HA-5 and HA-6 cements, respectively, as shown in Table 5.5. Similar effect on the cell viability was observed at the end of 48 and 72h. The cell viability values were higher than 75 % and 79 % after 48 and 72h as given in Table 5.6 and 5.7. Cytotoxicity results of the HA-4, HA-5 and HA-6 cultured for 24, 48 and 72 hours are shown in Figure 5.19.

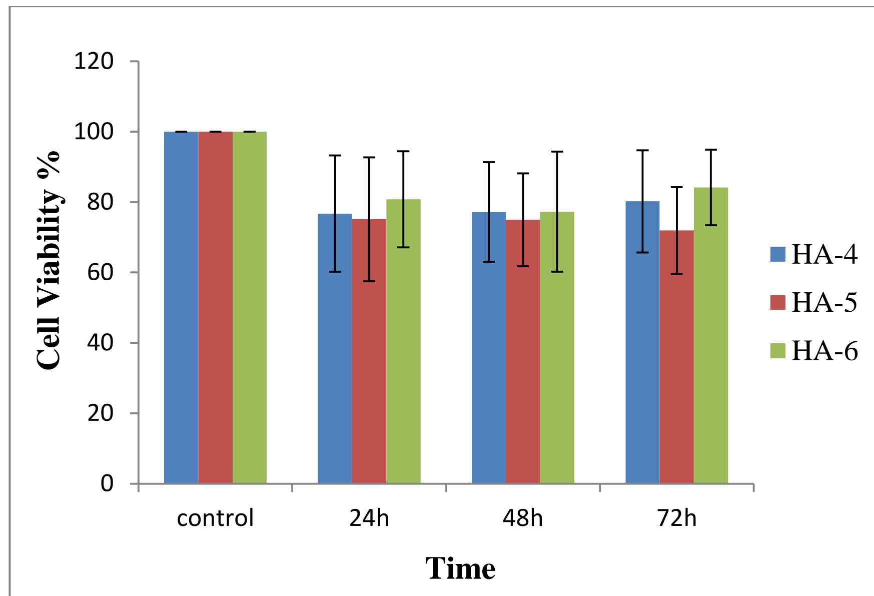


Figure 5.19. Cytotoxicity results of the HA-4, HA-5 and HA-6 cultured for 24, 48 and 72 hours.

The cell viabilities of HA-7, HA-8 and HA-9 cements (prepared by using 0.2M PB and 2.4 gr/mL S/L ratio) were found to be above 70 % after 24, 48, and 72 hours as given in Figure 5.20, Table 5.5, 5.6 and 5.7, compared to other cements. These cements showed slight decrease in the cellular viability at the end of 72 hours with respect to viabilities obtained after 24 and 48 hours.

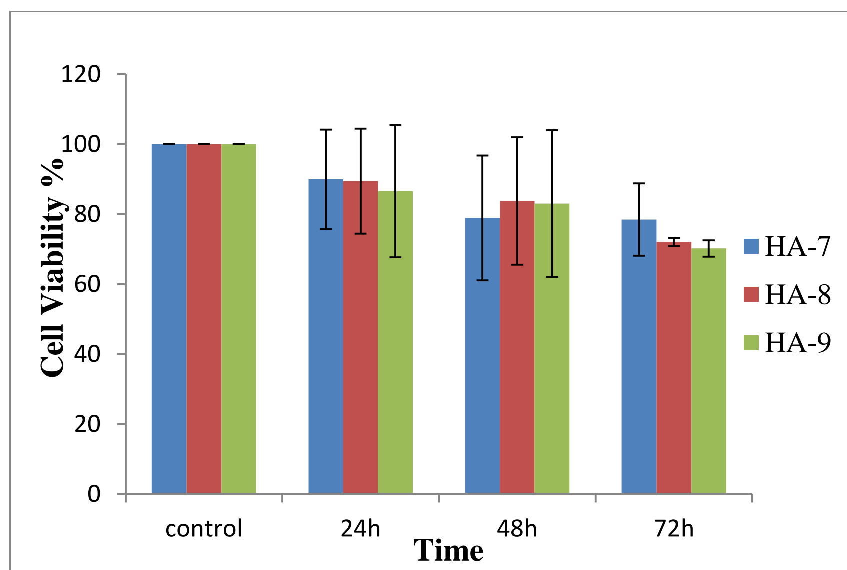


Figure 5.20. Cytotoxicity results of the HA-7, HA-8 and HA-9 cultured for 24, 48 and 72 hours.

The viability values obtained for HA-10, HA-11 and HA-12 cements were found to be 81 % and over for 24h, 76 % and over for 72 hours. However, a decrease in the cell viabilities were observed at the end of 72 hours compared to the viabilities obtained after 24 and 48 hours. Cell viability values of these three cements were found to be between 61 % and 65 % at the end of 72 hours as seen in Figure 5.21 and Table 5.5, 5.6 and 5.7. These results indicate that HA-10, HA-11 and HA-12 cements exhibit cytotoxic effect compared to the control. These cements were prepared using 0.3 M phosphate buffer. Solid/liquid ratio was 3.2 g/mL for all these cements. Among these cements only seed concentration varied. Compared to other cements, HA-1, HA-2, HA-3 were also prepared using 3.2 g/mL solid-liquid ratio but 0.2 M PB. Cell viabilities for HA-1, HA-2 and HA-3 cements were over 78 % in contrast to cell viabilities of HA-10, HA-11 and HA-12 (65-66 %). Two possible explanations might be postulated for the lower viability values obtained using HA-10, HA-11 and HA-12 cements at the end of 72 h. First, the redution of viability might be due to the use of more concentrated buffer solution during the preparation since the only variable was buffer strength (compared to the cements HA-1, HA-2 and HA-3). Second is the lower crystallite size of HA-10, HA-11 and HA-12 cements. Since their crystal structure were more closer to amourphous structure and they had lower crystallite sizes solubility of HA-10, HA-11 and HA-12 were possibly higher than HA-1, HA-2 and HA-3 cements. Cellular medium is not replaced with fresh medium during cytotoxicity testing. Due to the higher solubility of HA-10, HA-11 and HA-12 cements, concentrations of calcium and phosphate ions in the cell culture media were probably higher. It is likely that higher ion concentrations created in the cell culture media through the above stated structural and preparation originated reasons adversely affect the viability of cells.

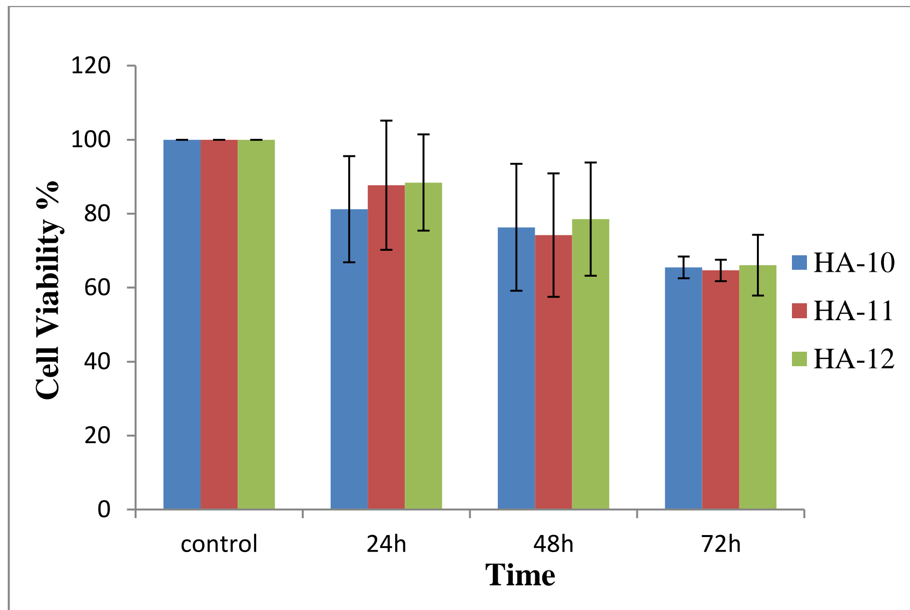


Figure 5.21. Cytotoxicity results of the HA-10, HA-11 and HA-12 cements cultured for 24, 48 and 72 hours.

HA-13, HA-14 and HA-15 prepared using 0.3M PB and 2.7 gr/ mL S/L ratio did not show toxic effects on the cell viability at the end of 24th, 48th and 72nd hours. However, there were slight decreases in the cell viability values at the 48 and 72 hours compared to vialibilites obtained after 24 hours. The cell viability values were found to be about 88% for 24h, 77% for 48h and 70 % for 72h as given in Figure 5.22 and Table 5.5, 5.6 and 5.7.

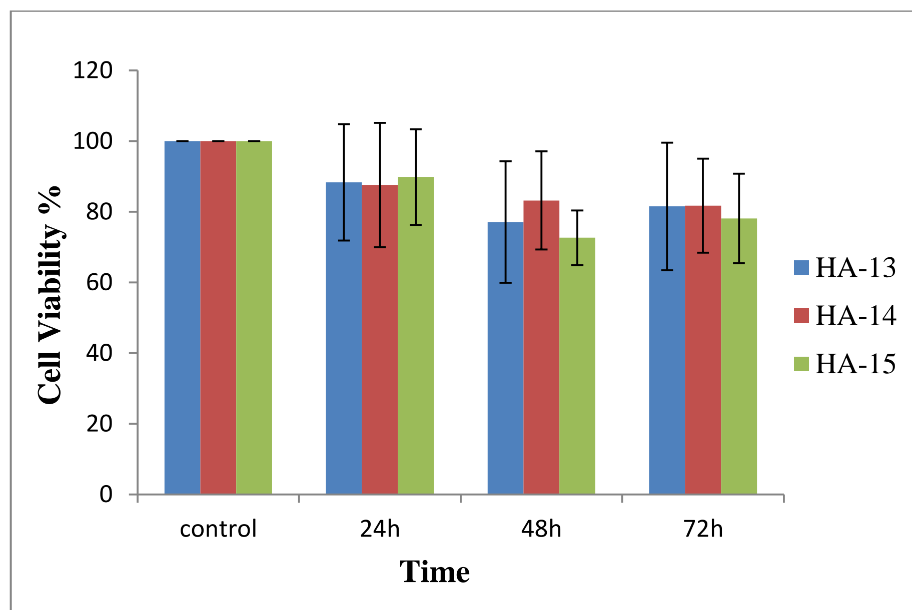


Figure 5.22. Cytotoxicity results of the HA-13, HA-14 and HA-15 cements cultured for 24, 48 and 72 hours.

HA-16, HA-17 and HA-18 cements did not show toxic effects on cell viability. Especially, the cell viability values of HA-17 and HA-18 cements were higher than 73 % for 24, 48 and 72h. The cytotoxicity results of the HA-16, HA-17 and HA-18 cultured for 24, 48 and 72 hours as shown in Figure 5.23 and Table 5.5, 5.6 and 5.7.

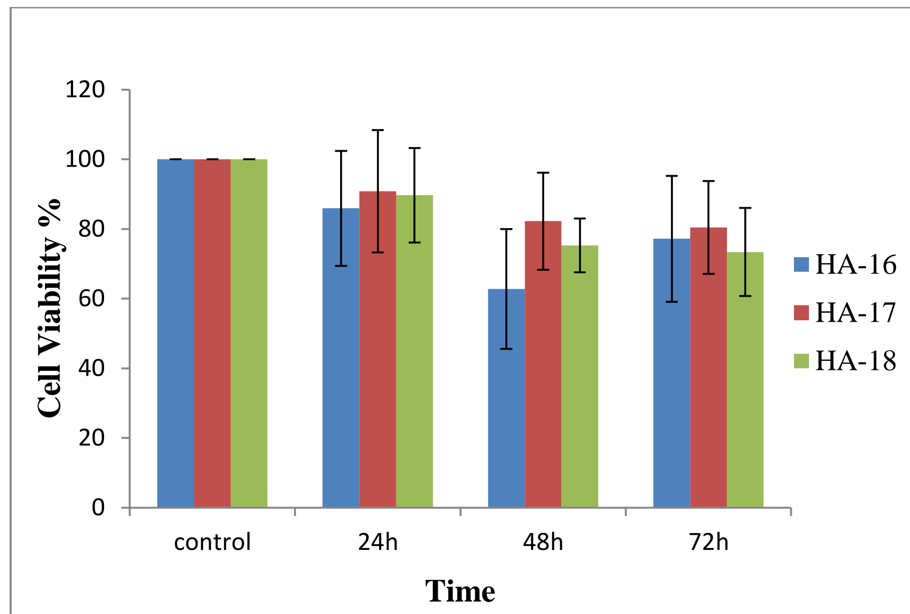


Figure 5.23. Cytotoxicity results of the HA-16, HA-17 and HA-18 cultured for 24, 48 and 72 hours.

Multiple response regression analysis were conducted on HA cement cytotoxicity results. Surface plots illustrate cell viability according to S/L ratio and seed concentration. According to Figure 5.24 and Table 5.5, the maximum cell viability values were seen in the HA cements prepared using 0.2M PB solution 2.4 S/L ratio and 1.5 % seed concentration at the end of 24 h. In an evaluation of cell viability results of HA cements prepared using 0.2M PB solution after 24 hour, it was seen that seed concentration was not an effective parameter on cell viability. Generally, the cell viability results of all HA cements at the end of 48 hours were slightly lower than 24 hours. Cell viability results of HA cements prepared with 0.2M PB solution after 24 and 48 hours are given in Figure 5.24 and Figure 5.25.

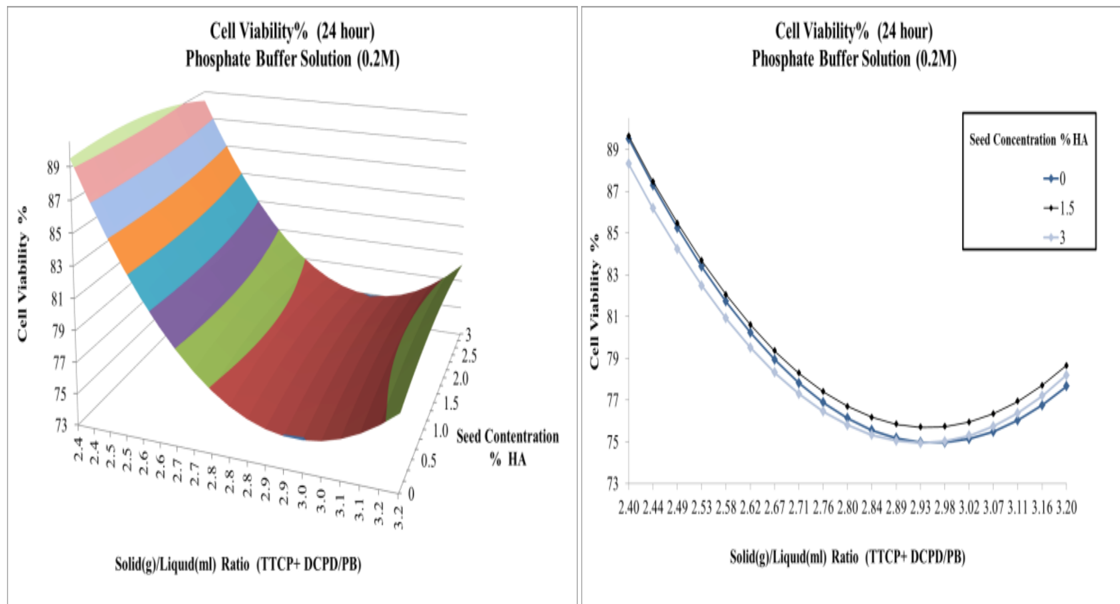


Figure 5.24. Cell viability results after 24 hour of HA cements prepared with 0.2M PB solution.

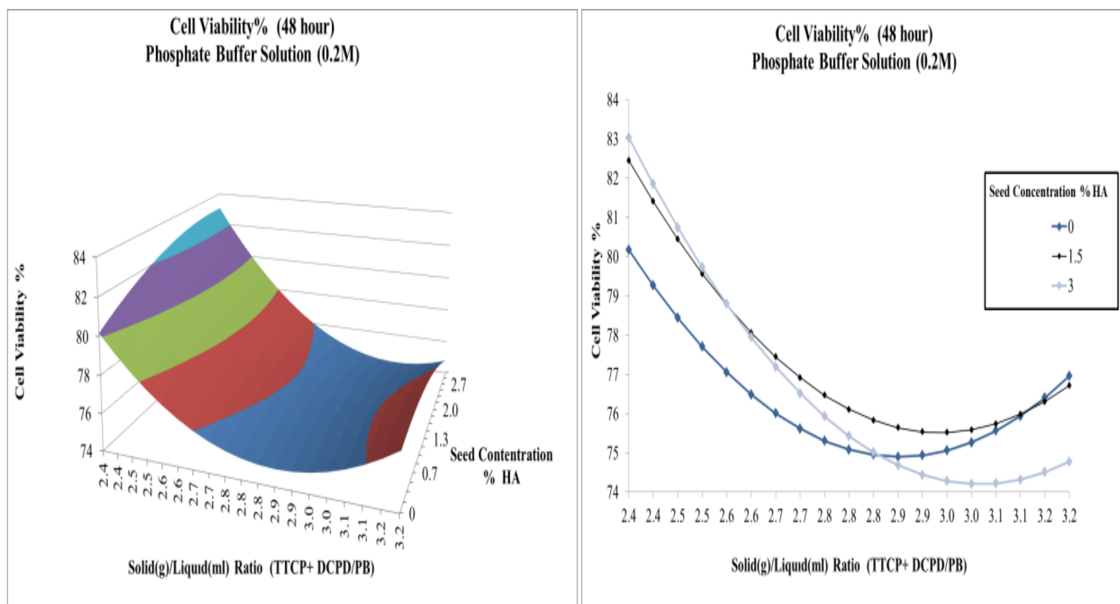


Figure 5.25. Cell viability results after 48 hour of HA cements prepared with 0.2M PB solution.

At the end of 72 hour, cell viability of HA cements prepared by using 0.2M PB solution tend to increase which also varies with seed content. According to the cell viability results of 72 hours, 0 % seed concentration was more favorable for cell viability compared to other seed contents (1.5% and 3%) as shown in Figure 5.26.

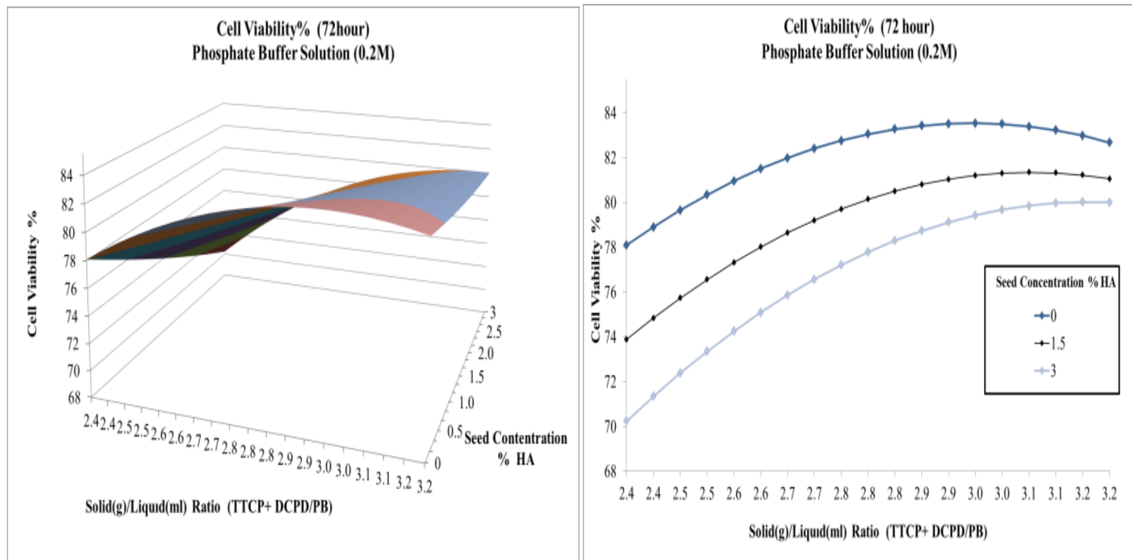


Figure 5.26. Cell viability results after 72 hour of HA cements prepared with 0.2M PB solution.

The viability values of cements prepared by using 1.5 % seed content and 0.3M PB solution was maximum at the end of 24h. Cell viability surface plot of HA cements prepared using 0.3M PB solution after 24 hour is shown in Figure 5.27. At the end of 48 and 72 hours, the maximum cell viaibilty values were seen in the HA cements prepared using 0.3M PB solution and 3% seed concentrations compared to the other seed concentration (1.5% and 0%). Cell viability surface plots of HA cements prepared with 0.3M PB solution after 48 and 72 hours are given in Figures 5.28 and 5.29.

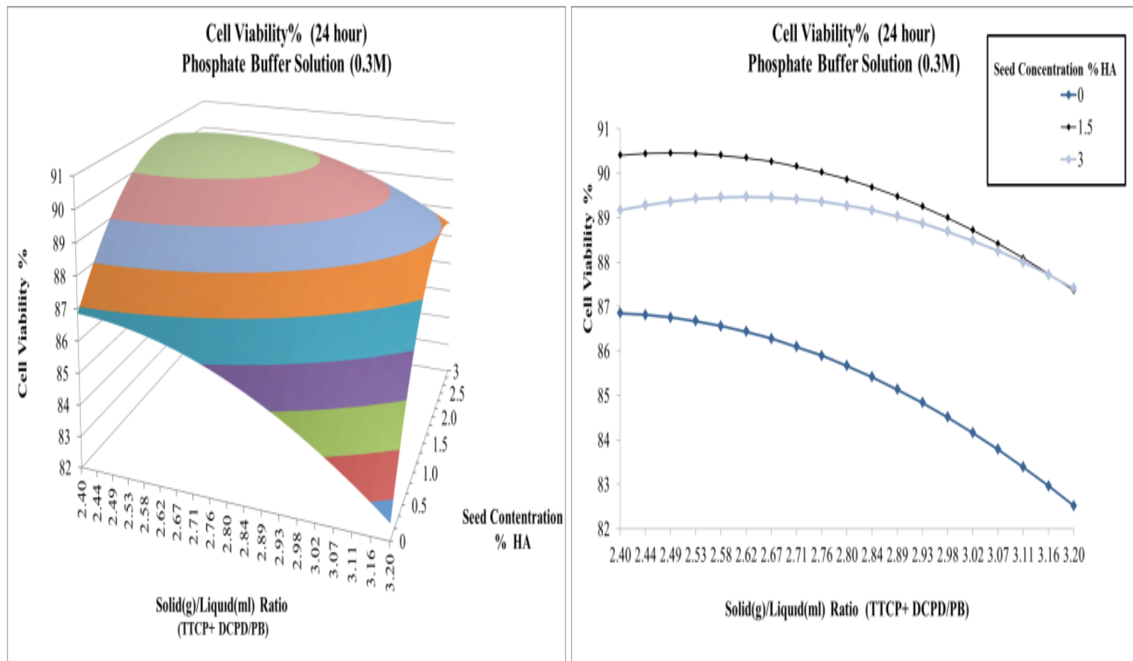


Figure 5.27. Cell viability results after 24 hour of HA cements prepared with 0.3M PB solution.

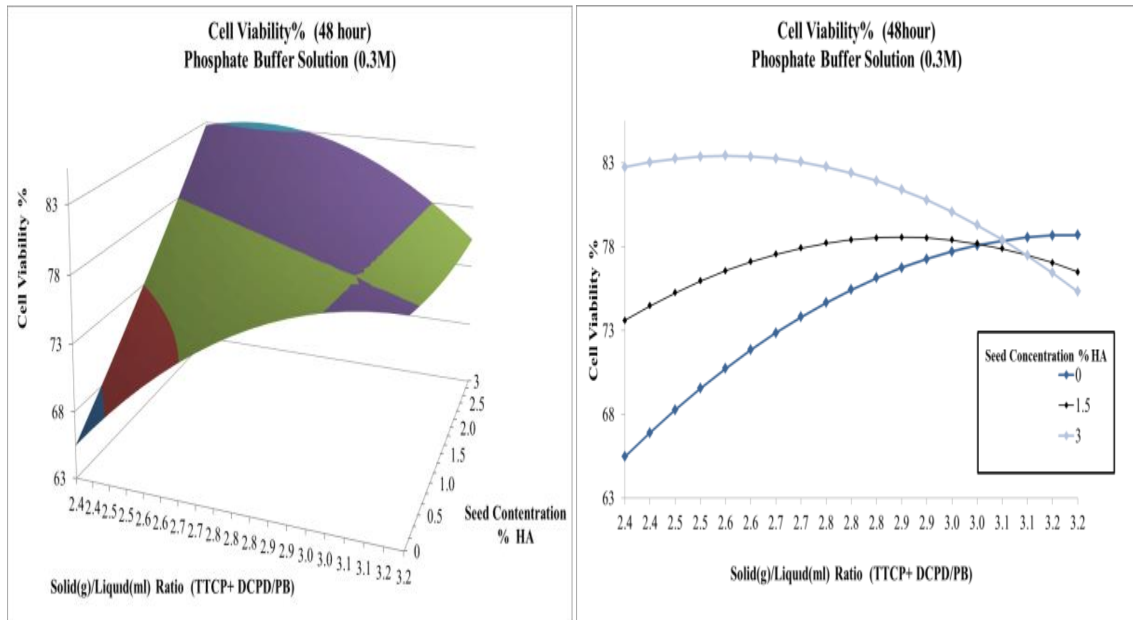


Figure 5.28. Cell viability results after 48 hour of HA cements prepared with 0.3M PB solution.

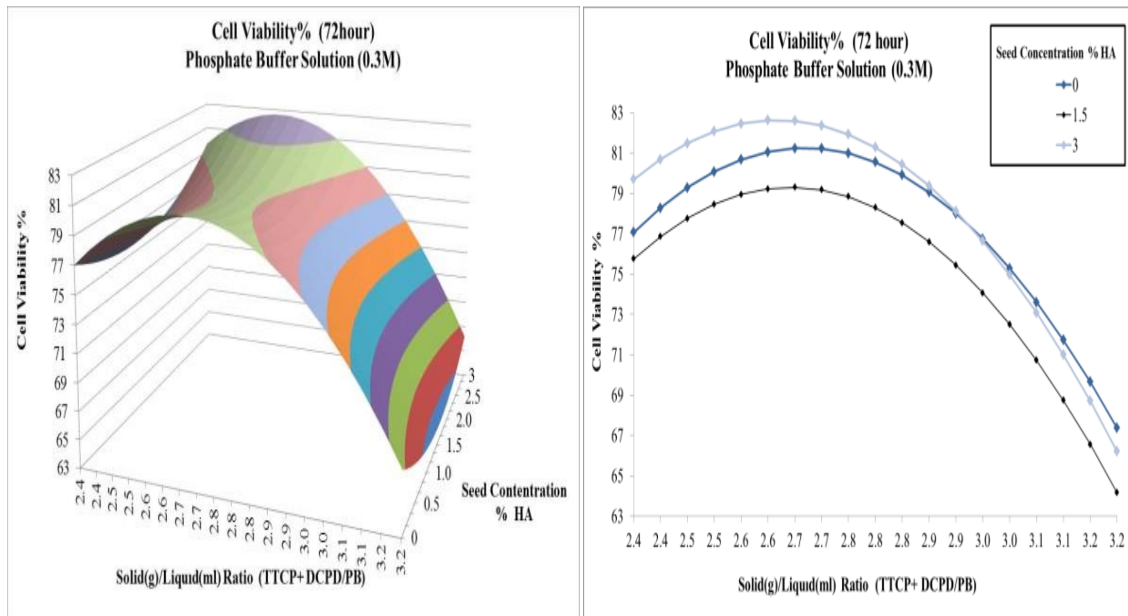


Figure 5.29. Cell viability results after 72 hour of HA cements prepared with 0.3M PB solution.

The cytotoxicity results of HA cements obtained in this study, as mentioned in the literatüre, are consistent with the cytotoxicity results found of Dagang and colleagues (2006). They investigated that cement prepared by mixing of TTCP and DCPA powders by using different concentrations of physiological saline or phosphate acid. They observed that all extracts exhibited no or low cytotoxicity. Calcium phosphate cement (CPC) preperad by saline had higher relative growth rate than CPC prepared by phosphate acid.

Similarly, according to the cytotoxicity results found by Guo et al. (2009), it was shown that all calcium phosphate cement (CPC) prepared by mixing of TTCP and DCPA powders by water exhibited no or low cytotoxicity after MTT assay. It was found that the cement samples did not exert any cytotoxic effects.

In another study (Lua et al. 2011), it was shown that magnesium doped apatite cement (md-AC) formed by adding MgO and calcium dihydrogen phosphate into TTCP/DCPA system apatite cement was biocompatible with no obvious negative effects on cellular growth. The cytotoxicity results obtained in this study were in good agreement with the literature data stated above.

Table 5.2. Cell viability values of L929 cells after 24 hours of brushite, TTCP-1 and TTCP- 2 powders.

Powders	**% Cell viability**			
Brushite	**% Dilutions**			
	100	**50**	**25**	**10**
	39	52	56	82
TTCP-1	81	80	85	85
TTCP-2	74	72	78	80

Table 5.3. Cell viability values of L929 cells after 48 hours of brushite, TTCP-1 and TTCP-2 powders.

Powders	**% Cell viability**			
Brushite	**% Dilutions**			
	100	**50**	**25**	**10**
	60	70	83	87
TTCP-1	83	87	90	92
TTCP-2	82	90	100	97

Table 5.4. Cell viability values of L929 cells after 72 hours of brushite, TTCP-1 and TTCP-2 powders.

Powders	**% Cell viability**			
Brushite	**% Dilutions**			
	100	**50**	**25**	**10**
	44	75	78	89
TTCP-1	87	96	90	97
TTCP-2	82	90	100	97

Table 5.5. Cell viability values of L929 cells after 24 hours of HA cement samples.

Solid/Liquid ratio	Buffer					
	0.2 M			0.3M		
	Cell viability %			Cell viability %		
	Seed % 0	Seed % 1.5	Seed % 3	Seed % 0	Seed % 1.5	Seed % 3
2.4	87	80	89	86	90	91
2.7	77	80	75	88	90	87
3.2	77	78	78	81	88	88

Table 5.6. Cell viability values of L929 cells after 48 hours for HA cement samples.

Solid/Liquid ratio	Phosphate Buffer					
	0.2 M			0.3M		
	Cell viability %			Cell viability %		
	Seed % 0	Seed % 1.5	Seed % 3	Seed % 0	Seed % 1.5	Seed % 3
2.4	79	75	84	62	75	82
2.7	77	77	75	77	77	83
3.2	77	76	76	76	78	74

Table 5.7. Cell viability of L929 cells after 72 hours for HA cement samples.

Solid/Liquid ratio	Buffer					
	0.2 M			0.3 M		
	Cell viability %			Cell viability %		
	Seed % 0	Seed % 1.5	Seed % 3	Seed % 0	Seed % 1.5	Seed % 3
2.4	78	72	72	77	73	80
2.7	80	84	79	72	78	82
3.2	83	78	81	65	61	65

CHAPTER 6

CONCLUSIONS

The aim of this study was to investigate cytotoxicity of HA cements prepared by using tetracalcium phosphate and brushite powders. In this context, TCCP and brushite powders were first chemically synthesized and characterized. Eighteen different HA cements were prepared by mixing the powders with 0.2M and 0.3M sodium phosphate buffers at pH 7.2. Setting times and cytotoxicity of the prepared cements on L929 mouse fibroblast cells were evaluated.

SEM images showed high temperature synthesized characteristic particle morphology of TTCP. Brushite powder had the typical microstructure of thick platelets of the brushite crystals. HA cements illustrated flower-shaped HA crystals.

The phase compositions of brushite, TTCP-1, TTCP-2 powders and HA cement samples were characterized by XRD. The analysis of XRD showed the presence of the characteristic peaks of brushite, TTCP and HA. X-ray diffraction patterns of brushite, TTCP-1, TTCP-2 powders and HA cement were in good aggrement with the JCPDS card numbers-72-0713, 70-1379 and 72- 0713, respectively.

According to the setting times of the HA cements, HA cements set in the range of 3.5 minutes and 20 minutes and converts to solid HA. HA-2 prepared with 0.2 M PB solution, 3.2 g/mL S/L ratio and %3 seed concentration showed the shortest setting time (3.5 min). HA-16 prepared using 0.3 M PB solution, 2.4 g/mL S/L ratio and 0% seed concentration had the longest setting time (24 min). Except for HA-11, % 3 seed concentration was found to be more effective on the shortening of setting time compared to the other seed concentrations (%1.5 and %0 seed).

As a result of the cytotoxicity of powders, brushite caused sharp decrease in cell viability at the end of 24, 48 and 72 hours at all concentrations examined. These results indicated that brushite powder had toxic effect on the cell viability especially, at 50% and 100% concentrations compared to control. The cell viability values obtained using TTCP powder extracts indicated that TTCP powders were biocompatible with cell viability values of 72 % and over. These results have shown that TTCP-1 and TTCP-2 had no toxic effects on cell viability.

The cell viability results after 24 hours of HA cements prepared by using hydroxyapatite powder as a nucleation/crystallization agent showed that seed addition had no effect on cell viability. The cell viability of the cements prepared using 0.3 M phosphate buffer solution was slightly higher than cements prepared with 0.2M phosphate buffer solution except the viability values at the end of 72 h for HA-16, HA-17 and HA-18 cements.

According to the cell viability results after 72 hours of HA cements prepared using 0.2M PB solution, cements prepared using 0 % seed concentration were more effective on the cell viability compared to the other seed concentrations (1.5% and 3%). At the end of 48 and 72 hours, the cell viaibilty values of HA cements prepared using 0.3M PB solution and 3% seed concentrations were maximum compared to the other seed concentration (1.5 % and 0%).

In conclusion, almost all HA cements and extracts of powders, except for brushite powders exhibited no cytotoxicity, although there are some differences between cell viability values of HA cements. Cell viability values of the powder extracts at lower concentration are superior to that of the extract at higher concentration. The influence mechanism of the extracts of various cements on the cells is very complicated which involves in many factors, such as, chemical composition, pH values and concentration of extracts and incubation time. As a matter of fact, it is difficult to fully understand the influence mechanism. According to all these results, it can be said that the HA cements prepared in this study had a promising application prospect for bone repair.

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