A Thesis

entitled

Monetite Cement Composites for Orthopedic and Dental Applications

by

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Calcium Phosphate materials have been used as bone substitutes and drug delivery systems for decades. Their scaffolds are useful tools in tissue engineering, orthopedic applications, and drug delivery. Calcium Phosphate Cement (CPC) is a bio-cement that supports loading in the absence of bone. They exhibit a remarkable biocompatibility, bioactivity, and partial biodegradability. However, their poor mechanical properties and fracture toughness in comparison to bone make them inappropriate for load bearing applications. A variety of materials and fibers have been added to CPCs as reinforcements to increase their mechanical properties. The aim of this study was to produce a new augmented CPC, monetite cement (MC), by reinforcement, and increased mechanical properties. Monetite cements were produced by mixing calcium hydroxide $\text{Ca(OH)}_2$ powders with an aqueous setting solution. This product exhibits poor mechanical strength in comparison to cancellous bone. Carbon nanotubes (CNTs) and Chitosan, with their good mechanical properties both in tension and compression, have potential use in CPCs to further strengthen the mechanical properties. In this project, techniques and methods for synthesizing and making MC-CNT and MC-Chitosan have been compared. It was observed, the surface modification of CNT and
chitosan, to enhance bonding to monetite, is necessary in order to achieve good compressive strength. Strong interfacial bonding of CNT-COOH and deposition of biomimetic calcium phosphate on chitosan powders are necessary to improve the mechanical properties of MC-CNT and MC-Chitosan composites. The mechanical and biological performance of MC-CNT and MC-Chitosan composite cements were evaluated and show potential in orthopedic and dentistry applications.

The main ingredient of monetite cement is calcium hydroxide. Egg-shells have a considerable amount of calcium in their structure and provide a cheap renewable source of this mineral. If monetite cement could be produced from this natural resource it would create a new green technology saving tons of egg-shells from landfills each year. Mechanical and biological performance of monetite cement from eggshells is comparable to pure monetite cement.
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List of Abbreviations

ALP ................................................................. Alkaline Phosphatase
CaP ................................................................. Calcium Phosphate Materials
CDHA .............................................................. Calcium Deficient Apatite
CNT ................................................................. Carbon Nanotubes
CSD ................................................................. Calcium Sulfate Dihydrate
CSH ................................................................. Calcium Sulfate Hemihydrate
DCPA ............................................................... Dicalcium Phosphate Anhydrate (Monetite)
DCPD ............................................................... Dicalcium Phosphate Dihydrate (Brushite)
FBS ................................................................. Fetal Bovine Serum
FTIR ............................................................... Fourier Transform Infrared Spectroscopy
HA ................................................................. Hydroxyapatite
MC ................................................................. Monetite Cement
MCPM ............................................................ Monocalcium Phosphate Monohydrate
MEM .............................................................. Monocalcium phosphate monohydrate
PBS ................................................................. Phosphate Buffered Saline
SBF ................................................................. Simulated Body Fluid
SEM ............................................................... Scanning Electron Microscope
TCP ............................................................... Tricalcium Phosphate
XRD ............................................................... X-Ray Diffraction
Chapter 1

Monetite Cement for Biomedical Applications

1.1 Bone Substitute Materials

A serious rise in the number of musculoskeletal disorders, such as osteoporosis, osteoarthritis, and the number of medications to heal these diseases, requires the development of new drugs and active substances for treatment. Osseous substances are often required for filling defective parts in orthopedic surgery [1]. Bone repair is necessary for a variety of reasons. For example, fractures create voids or cracks which need filling. Sometimes, cancerous bone tissue needs removal and voids are created which need to be repaired. Moreover, regeneration of bone becomes more difficult because of factors like age, disease and trauma.

The most important issue for any biological implant is developing a biocompatible and porous material which speeds up the development of local bone formation[2]. Self-hardening and injectable Calcium Phosphate Cements (CPCs) are potential materials for bone defect repair in surgery due to their similarity to the mineral phase of bone which contains calcium and phosphate. Additionally, CPCs possess great biocompatibility and excellent osteoconductivity[3].
Most Calcium Phosphate based orthopedic cements available today set into calcium hydroxyapatite [HA, Ca_{10}(PO_4)_6(OH)_2] as their final product. This is useful because of the similarity of HA with the mineral portion of human bone\[4\]. Among all the calcium phosphate phases HA has the lowest solubility\[5\]. Moreover, in vivo research on HA based cements shows that HA resorbability is very low, and there is less than full participation in the bone remodeling processes even a year after implantation\[6, 7\]. HA-based cements usually use α-tricalcium phosphate [α-TCP, α-Ca_3(PO_4)_2] or tetracalcium phosphate [TTCP, Ca_4(PO_4)_2O] as their major constituents\[4\]. Hydrolysis of both these phases forms apatitic calcium phosphates, when they are soaked or in contact with aqueous solutions.

There are some problems with α-TCP or TTCP powders requiring appropriate facilities. First of all, they cannot be synthesized at room temperature and are stable only at high temperatures around 1200°C\[8\]. Consequently, these high-temperature phases must be quenched to the ambient temperature, by using quite capital intensive manufacturing processes \[8-10\]. The involvement of high temperatures in synthesizing α-TCP and TTCP powders also places a burden on the particle size of these important cement ingredients. Typically, quenched chunks or hard agglomerates of these materials require a delicate grinding operation, which may be a further source of contamination, or premature and undesired interaction of the powders with humidity present during grinding. As a result, the reactivity of these powders would also be a major point of concern\[11\].
CPC contains two phases: solid phase and liquid phase. Powder in solid phase contains some elements of calcium and phosphate salts. Water and an aqueous solution are the components of the liquid phase[12].

As far as mechanical properties go, CPCs are ceramics, and therefore brittle materials. This limits their usefulness in various stress-bearing locations or unsupported defects[2]. Low strength causes the cement to fail or collapse under low stresses.

Another deficiency of using CPCs is their long setting time [13]. A long setting time can result in the breakdown of CPC when the cement, before its setting completes, comes in contact with fluids or blood[14]. Because of these issues, many researchers have tried to improve these properties by adding small amount of various materials and fiber as reinforcement to enhance the operation of this cement[15].

CPCs have low compressive strength which led to make ceramic composites with reinforcements materials to improved strength and fracture toughness[16]. Poor tensile strength of CPCs as compared with bone makes it inappropriate for load bearing locations[17]. These days, using fibers, chitosan, and CNTs in CPCs has become common because of the improved mechanical properties of reinforcement materials. Fibers, chitosan and CNTs as reinforcement phases increase the strength and toughness of CPCs without counterbalancing bioactivity and biodegradability of the product[3].

1.2 Development of calcium phosphate cements

During the 1960s and 1970s, Posner and co-workers[18] dedicated their research to defining the mineral of bone. Some studies were completed by these researchers and a variety of reports published by them led to a very good understanding of the structure of bone[19, 20]. According to their reports, bone has a very complex three dimensional
structure composed of organic and inorganic elements. The organic constituent is mainly collagen (TYPE I) along-with certain organic acids like citrates and lactates[21]. The inorganic constituent is a continuously changing apatitic structure, the composition of which varies according to location and the age of a person. Essentially, it is a poorly crystallized, carbonated apatitic calcium phosphate[22]. At various places, the divalent Ca\(^+\) ion is substituted by other alkali ions like K\(^+\), Na\(^+\), and Mg\(^+\). Since HA or hydroxyapatite resembles the inorganic part of bone, it was one of the very first successful artificial materials used in healing and bone repair[23, 24].

HA implants were the very first treatment for bone repair[23]. Implants play an important role in healing the broken bone in serious injuries. Because implants have limited applications due to incomplete filling of the bone defects, the need for a moldable self-setting implant which could be applied by hand or injected by a syringe, was realized[5, 25, 26]. These implants were synthesized by high temperature processes which lead to dense, sintered particles[27, 28]. In some cases, particles were crushed and ground and subsequently used to fill these defects[29]. Additionally, it was the start of use in dental applications such as root canal filling [30, 31]. HA is biocompatible and also binds well to natural bone. Cells attach and proliferate on these implants[32, 33]. In bio-reabsorption \(\alpha\)-TCP and \(\beta\)-TCP have a higher solubility product as compared to HA which incited its use as an implant for orthopedic repair [34, 35].

Implants made of calcium phosphate components had limited applications due to partial filling of the bone defect[36]. Due to this problem, the need for a moldable self-setting implant which could be either applied by the hand or injected using a syringe was realized[37, 38]. Brown and Chow invented the first Calcium Phosphate (CaP) self-
setting cement, which could be used as a paste to fill the bone defect, subsequently hardening in-situ to form HA[37, 38]. The powder component of this cement formulation was a mixture of TTCP and DCPA. In-vitro and in-vivo experiments showed the cement to be biocompatible. Initially, the liquid component of these cements was reported to be deionized water (DI-Water), however, it was later modified to a dilute sodium phosphate solution[29, 30]. HA formation in cement formulations using TTCP and other CaP materials was also demonstrated[39, 40].

The first CPC was produced in 1986[14, 41]. Many researches in various laboratories all over the world have tried many different compositions and diverse methods of mixing CPCs to come up with useful compositions and optimize results. The old components have other benefits such as low setting temperature, ease of formation into arbitrary geometries and good stiffness. Also, these cements have led to the production of a new class of ceramic grafting material, which are moldable, set at physiological conditions, and therefore find applications in non load bearing locations. They can be used in dental, craniofacial and periodontal practices to repair bone defects[38]. This also has found applications in orthopedics[42, 43].

After the work of Brown and Chow[37], there have been reports of many CaP cement formulations aimed at different final stoichiometry’s for various clinical applications. Bohner et. al[44] reviewed the most recent achievements in the field of CaP cements[45]. There are three main classes of CaP cements currently, (a) those which use TTCP as the main component of the powder[37, 46], (b) those using α-TCP as the main component of the powder[47, 48], and (c) those using β-TCP as the main component of the cement powder[49, 50]. Brown and Chow’s[39] recipe was about increasing
compressive strength values and decreasing porosity by compaction of pastes which Barralet[51] and co-workers reported. The same authors reported amplified compressive strength values in apatitic cements based on TTCP and α-TCP by chemical additions to the liquid component of the cement formulation[52, 53]. Ionic modification of CaP cement viscosities also lead to improved strengths and injectability for apatite and DCPD based cements with potential use in fixation of implants[54-56]. Barralet et. al[57] have also reported that DCPD and pyrophosphate cements, using nanocrystalline hydroxyapatite and β-TCP respectively as the starting materials, have good potential bone-replacement applications[57-59].

Although HA based cements demonstrated good biocompatibility and strength post setting, their main problem was their inability to reabsorb in the body[60, 61]. From various studies, solubility of HA product is quite high, hence its decreased bioresorbability[34, 35]. Lemaitre and Mirtchi[62] invented a DCPD cement based on β-TCP as the main component of the powder. This cement demonstrated good biocompatibility in-vivo[50, 63]. Phosphoric acid or MCPM was used in combination with β-TCP and the setting solution consisted of de-ionized water, Na₂HPO₄ solution, or a hyaluronic acid solution[64, 65]. Since DCPD is a metastable phase, known for its quick conversion to apatite in-vitro, these cements were much faster resorbing compared to apatitic cements as shown by several studies[66-69]. In some cases, it was found that before getting resorbed, DCPD cements after implantation transformed to apatite, which reduced their resorbability[69].

Similarly, α-TCP in combination with DCPD or DCPA is known to have self-setting properties to form Calcium Deficient Apatite (CDHA) as the end product [13, 70,
Recently, a new class of cements combining Calcium Sulfate Hemihydrate (CSH) or Calcium Sulfate Dihydrate (CSD) with α-TCP to form CSD and CDHA as the end-products has been reported[44, 72, 73]. Since CSD is similar in composition and structure to DCPD, it may be incorporated in the cement without appreciable loss of strength while maintaining its resorbability[73].

Currently, CaP cements are only available in two groups consisting of hydroxyapatite (HA) and dicalcium phosphate dihydrate (DCPD, CaHPO$_4$.2H$_2$O) or brushite, based on the final phase of the set cements. Recent interest in the development of CaP cements is shifting more towards monetite (dicalcium phosphate dihydrate, DCPA, CaHPO$_4$) cements due to their proven high bicompatibility. The bioresorption rate of monetite cements, i.e., their capability to take part in the in vivo bone remodeling processes was found to be significantly more than apatitic cements and less than HA[74].

However, all of the currently known cement formulations were based on a powder component which consists of an intricate mixture of at least two or more CaP or Ca- or P-containing phases, usually some of those ingredients are basic and the remaining are acidic in nature. Upon achieving the first contact with the setting solution, neutralization reactions start to take place in the powder components to form the apatite- or monetite-like end phases. Such a mixture of CaP may undergo a solid state reaction, limiting the shelf life of these cements and also their storage conditions[75].

1.3 Disadvantages of current CPCs

Current CPCs have some deficiencies in biomedical applications. Some of the CPCs have restricted re-absorbability in-vivo. This limitation makes it unsuitable for
certain applications[76, 77]. In addition, apatite inhibits Alkaline Phosphatase (ALP) activity, which is a marker of cell differentiation during mineralization is stable to the process of bone-remodelling[78, 79]. DCPD cements have shown better bio-absorbability in-vivo although increasing the inflammatory response in some cases which could be attributed to their acidic nature[77, 80-82]. Another problem with current CPCs formulations is most of them contain a mixture of two or more chemicals as their powder component, which might reduce their batch to batch reproducibility and shelf life[83].

There are some problems that all current CPCs have. For instance, they are cost prohibitive, time consuming and hard to produce. All current cements are based on CaP starting materials like TTCP or TCP which can only be synthesized at high temperatures. These high temperatures cause sintering and make the material dense. They also have an effect on their reactivity and make them less reactive. Such dense, sintered blocks are ground to form powders, which are subsequently used in the cement recipes. The grinding step causes impurities to be incorporated in these raw-materials. It is also very hard to grind these dense and sintered blocks. In many cases, the dense starting material remains as is in the form of particles or granules even after the cement has set. Such dense particles or granules may lead to local differences in absorption rates in-vivo since absorbability for such dense sintered blocks or granules is less[27].

1.4 Components of the thesis

This thesis is divided into focal points with regards to monetite cement composites in orthopedic and dental applications. The first section describes a new method to produce monetite cement with augmentation of surface modified carbon
nanotubes. Surface modification provides essentials bands between carbon nanotubes and cement. The results of the material synthesis and biocompatibility study followed by tables and figures are contained in Chapter 2. The remaining portion of the thesis describes the synthesis of surface modified low molecular chitosan particles in monetite cement to increase mechanical properties of this product. In this part of project, surface modification of chitosan particles to prepare bonding between chitosan particles and monetite cement. The results of all experiments have done are contained in Chapter 3. In Chapter 2 and Chapter 3, Calcium hydroxide was used in the cement because it is an inexpensive source of calcium.

Chapter 4 describes a new method for producing monetite cement from natural resources. In this chapter preparing calcium hydroxide powder from eggshells and synthesizing the cement from this product is mentioned.
Chapter 2

Effects of Multi-walled Carbon Nanotubes on the Mechanical Properties of Monetite Cement

2.1 Introduction

2.1.1 Carbon Nanotubes

Carbon nanotubes (CNTs) are allotropes of carbon with cylindrical nanostructures. They are a new form of carbon with unique mechanical, electrical and magnetic properties[84]. Nanotubes have been constructed with length-to-diameter ratio of larger than any other material[85]. These cylindrical carbon molecules have unusual properties, which are valuable for nanotechnology, electronics, optics and other fields of materials science and technology. In particular, owing to their extraordinary thermal conductivity and mechanical and electrical properties, carbon nanotubes find applications as additives to various structural materials. For instance, in (primarily carbon fiber) baseball bats, golf clubs, or car parts, where nanotubes form only a tiny portion of the material[86]. CNTs are one of the strongest materials that will ever exist with a tensile strength greater than steel, but only six times lighter the weight of steel[87].

Nanotubes are categorized as single-walled nanotubes (SWNTs), which considered as the results of folding graphite layers into carbon cylinders and maybe
composed of a single shell or single wall nanotubes, and multiwall carbon nanotubes (MWCNTs) which contain several shells or walls. Individual nanotubes naturally align themselves into ropes held together by van der Waals forces, more specifically, π-stacking.

Iijima in 1991 was the first person who discovered carbon nanotubes (CNTs) using arc discharge method[88, 89]. Following this discovery, a number of scientific research projects have been initiated and a variety of methods have been used to synthesize CNTs, namely, arc discharge, laser vaporization and catalytic chemical vapor deposition of hydrocarbons[90-92]. Since carbon–carbon covalent bonds are one of the strongest in nature, a structure based on a perfect arrangement of these bonds oriented along the axis of nanotubes would produce a remarkably strong material. Nanotubes are strong and flexible structures that can be bent and stretched into shapes without catastrophic structural failure in the nanotube[93, 94].

Most single-walled carbon nanotubes (SWCNT) have a diameter of close to one nanometer, with a tube length that can be many millions of times longer. The structure of a SWCNT can be conceptualized by wrapping a one-atom-thick layer of graphite called graphene into a seamless cylinder. The way the graphene sheet is wrapped is represented by a pair of indices (n,m). The integers n and m denote the number of unit vectors along two directions in the honeycomb crystal lattice of graphene. If m = 0, the nanotubes are called zigzag nanotubes, and if n = m, the nanotubes are called armchair nanotubes. Otherwise, they are called chiral.

Multi-walled carbon nanotubes (MWCNT) consist of multiple rolled layers (concentric tubes) of graphene[95]. Their morphology and properties are similar to those
of SWCNT but their resistance to chemicals is significantly improved[96]. This is especially important when functionalization is required. This means grafting of chemical functions at the surface of the nanotubes to add new properties to the CNT. In the case of SWCNT, covalent functionalization will break some C=C double bonds, leaving "holes" in the structure on the nanotube and, thus, modifying both its mechanical and electrical properties.

2.1.2 Using Carbon Nanotubes in Monetite Cement

CNTs have potential to be used in bone regeneration applications. The benefits of using CNTs in Monetite Cements (MCs) as reinforcement phase are known for more than decades[97]. But there is no work has been done about augmentation of monetite cements with CNTs.

As far as mechanical properties go, MC is a ceramic, and therefore a brittle material. This leads to the creation of a thin bone limiting its usefulness in various stress-bearing locations or unsupported defects[2]. Low strength causes the cement to fail or collapse under low stresses.

One of the main purposes of making ceramic composites with reinforcements is to make a material with improved strength and fracture toughness[16]. Improving MC by adding small amount of various materials and fiber as reinforcement to enhance the operation of this composite is the objective of this study. Poor tensile strength of MC as compared with bone makes it inappropriate for load bearing locations[17]. CNTs as reinforcement phase increases the strength and toughness of MC without counterbalancing bioactivity and biodegradability of the product[3].
2.2 Materials and methods

2.2.1 Cement Powder and Liquid

The cement powder consisted of a mixture of calcium hydroxide (Ca(OH)$_2$=74.10 g/mol, BDH Laboratory Supplies, Poole, UK), CNTs as the reinforcement, setting solution, and water.

2.2.2 CNTs

The multi-walled carbon nanotubes (COOH Functionalized MWCNT; 10-20 nm OD 95% 5-10 nm 10-20 nm 10-30 μm>200 m2/g Surface carbon functionalization ratio: 8-20 mol%, weight percentage: 2.0 wt% .22 g/cm3 ~ 2.1 g/cm3 black) were used in this study.

Using CNTs in MC instead of increasing the mechanical properties, drop the strength of the cement because there is no bonding between cement particles and CNTs. There is an important issue about enhancing bonds between carbon molecules and the cement molecules. This issue has been solved by modifying the surface of CNTs with a bond of COOH to attach to calcium molecules and makes the cement stronger.

In the first step for preparing surface modified CNTs, impurities were removed by synthesizing CNTs with concentrated sulfuric acid (H$_2$SO$_4$, Fisher Scientific, Fair Lawn, NJ) and nitric acid (HNO$_3$, Fisher Scientific, Fair Lawn, NJ) volume 3:1. The mixture was ultrasonicated (power of 100 W and nominal frequency of 50 kHz at ambient temperature) for two hours, and then magnetically stirred at 70°C for three hours. After the mixture was cooled to room temperature, it was diluted with deionized water and then
poured in centrifuge tubes and centrifuged with centrifuge machine (3000 rpm for three min), and washed with distilled water until the pH value of the filtrate was around seven. Sediment was dried in the furnace at 70°C, giving the CNT-COOH. CNT-COOH now easily attach to calcium molecules of MC[98].

2.2.3 Setting Solution

Setting solution for the cement samples was prepared by mixing different reagents and deionized (DI) water. For preparing 15ml of the setting solution, 0.0032gm of citric acid monohydrate (CAM, 100% assay, C₆H₈O₇.H₂O, Fisher Scientific, Fair Lawn, NJ) which encourages the formation of Brushite, 6gm of sodium bicarbonate (NaHCO₃>99.7%, Fisher Scientific, Fair Lawn, NJ) which causes setting of the cement, 1.95ml of DI water for diluting the setting solution, and 13.05ml of phosphoric acid (85%H₃PO₄, EMD Chemicals Inc., Gibbstown, NJ) as a source of phosphate in the cement, were mixed respectively. It is important to consider that adding the phosphoric acid should be done by titrating droplets because the solution is highly reactive. All setting solutions were prepared in clean glass beakers and the order of addition of each reagent starting from citric acid to phosphoric acid respectively. Setting solutions were stored in tightly-capped glass bottles. Throughout this study, the pH stability of the setting solutions was monitored over a period of six months.

2.2.4 Cement Synthesis

Cement pastes were prepared by manually mixing calcium hydroxide with setting solution, DI water and CNTs in an agate mortar by using an agate pestle. The mixing ratios of Ca(OH)₂ and the setting solution were initiated according to the Ca/P molar
ratios to be reached in the final paste. Since the goal of this study was producing CaHPO₄-based pastes and keeping away from the formation of apatitic calcium phosphates as much as possible, the Ca/P molar ratio of one was studied.

Initially, 0.6175gm of Ca(OH)₂ mixed at least two minutes with 0.8ml of DI water to disperse the Ca(OH)₂ completely in the water. The dispersion should be long enough to avoid any agglomeration of calcium hydroxide particles.

Optimized concentrations of water are important to get the most favorable strength and setting time. Also, by optimizing the amount of water, injectability of the cements by a needle can be improved.

After dispersion step, different ratios of surface modified CNTs were added to the sample and again mixed with an agate pestle to completely disperse all of the CNT particles in the solution. The color of the solution after adding surface modified CNT gradually from white to grey and finally turned to dark grey.

Finally, 0.75ml of setting solution was added to the materials above and placed in the mold to make it a disk sample for compression test and cell culture. Before these tests, the samples were dried in a furnace for four hours and after that they were soaked in DI water for 24 hours before testing to mimic body environment.

### 2.2.5 Setting Time

After preparing cement, the cement placed in the mold (12mm diameter and 8mm height) for completing setting time experiment by Gillmore needle apparatus (Humboldt Mfg. Co., Schiller Park, IL). Initial (tᵢ) and final (tᵢ) setting times of cement samples were determined by using Gillmore needle according to the international standard ISO 9917 for dental cement and ASTM C266-89[99, 100]. The Gillmore needle is an instrument
used in a penetration type of test for measuring the setting time of materials such as Portland cements, pastes, masonry cement, hydraulic hydrated lime and certain mortars. According to the American Dental Association specification No. 61115 complies with ASTM standards C91, C141, C150, C266, C414; AASHTO T154. The Gillmore needle has two stainless steel cylindrical flat-end needles. One of the needles which has a weight of ¼ lb (115.12 g) and the diameter of 1/12" (2.12mm) was used for initial setting time. The heavier needle has a diameter of 3/16" (4.8mm) and the weight of 1 lb (453.6 g) used for final setting time.

2.2.6 Mechanical Testing

Much published research [101, 102] focuses on the compressive strength of MC based cements because of the inability to fixture these cements for tensile tests. Therefore, compressive strength has been used in this study to derive mechanical properties. It is easy to get higher values of compressive strength by using a hydraulic press to firmly pack the cement paste during the setting process or sintering the powder in a high temperature furnace. These methods would not be practical in clinical applications because it would damage surrounding bone pressing the cement to reach the desire strength. On the other hand, by compacting the bone cement paste very tight during formation, the pore size of the cement particles is decreased. This causes reducing biocompatibility since cells would not be able to attach themselves into the cement scaffold[69]. The cement was compressed by hand at room temperature into cylindrical molds similar to as practiced by dental surgeons repairing caries.
The purpose of compression tests were to figure out the effect of reinforcements with various ratios on mechanical properties and strength of the MC-CNT specimens. MC-CNT specimens were tested at four different mass fraction of CNT in liquid: 0, 0.5, 1 and 2%. The mixed of MC-CNT paste were placed into a circular mold of 12mm diameter and 8mm height. Cylinder dimensions were carefully measured using a digital caliper. Before testing, each specimen were incubate in an incubator with 100% humidity at 37°C for 24 hours and finally soaked in DI-water at 37°C for 24 hours before testing. Disk specimens of the composite materials were tested to failure in uniaxial compression test using INSTRON Machine (50 KN capacity, Bluehill software package 2). Compressive load versus compressive extension plots and data were obtained from INSTRON machine too. The crosshead rate of loading, as American Dental Association suggested 0.75±0.25mm/min, was set to 0.5 mm/min. A minimum of six samples were built for each combination of reinforcement. The compressive modulus was determined from the slope of the linear region in the stress-strain diagram[43]. Strength and strain were calculated using the following equations.

\[
\text{Strength} = \frac{P_{\text{max}}}{A}
\]

\[
\text{Strain} = \frac{\text{max}}{\text{max}}
\]

Where \(\sigma_{\text{max}}\) is compressive strength, \(\varepsilon_{\text{max}}\) is ultimate strain, \(P_{\text{max}}\) is the peak load, \(A\) is area, \(D\) is diameter, and \(l\) is the height of the specimen.
Before the test, all of the sharp edges of samples were polished by polish paper to decrease the effects of stress concentration in sharp edges.

2.2.7 Composite Characterization

2.2.7.1 X-Ray Diffraction Analysis

The changes in the crystallographic structures of the MC-CNT produced were examined using X-Ray Diffraction (XRD, Rigaku Ultima III) using a speed of one degree per minute in continuous scan mode with 40 kV and 44 mA. The XRD data was collected for a 2θ range between 10° and 60°.

2.2.7.2 Morphology Observation of Cements

The morphological developments of the Monetite formed in the presence and absence of CNTs and soaked either in α-MEM or SBF were monitored using scanning electron microscope (SEM, Hitachi S-4800, Hitachi Corp, Tokyo). The samples were first dried out completely and mounted on conducting carbon tape, coated with gold and were visualized with accelerating voltage of 10 KV, magnification of 1.00 K.

2.2.7.3 Fourier Transform Infrared Spectroscopy Analysis

The Fourier Transform Infrared Spectroscopy (FTIR, UMA-600 Microscope, Varian Excalibur Series) was conducted on particles synthesized using Ca/P ratio of one.
2.2.8 Cell Culture Experiment

Cell Culture was done on hardened monetite and MC-CNT samples with MC3T3 cells at 37°C with 100% relative humidity and 5% CO₂ in complete α-MEM. 100 ml of complete α-MEM contains 90 ml of α-MEM (HyClone MEM Alpha Modification 1X, Thermo Scientific, Logan, UT), 10 ml of fetal bovine serum (HyClone FBS, Thermo Scientific, Logan, UT) and 100 μl of antibiotic (HyClone Antibiotic, Thermo Scientific, Logan, UT).

MC3T3 cells were grown on 75 cm² culture flasks (BioLite 75 cm² Flask, Thermo Fisher Scientific, Rochester, NY) at 37°C and 5% CO₂ in complete α-MEM. The culture media was changed every two days until the cells reached a convergence of 90 - 95%, as determined visually with an inverted microscope. The cells were then detached from the surface of the flask using trypsin (2.5 g/L, EDTA 25mM solution, Sigma-Aldrich Corp., St. Louis, MO). The obtained cells were then seeded at a concentration of 10000 cells per well for various assays. MC-CNT pellets measuring 12 mm in diameter and 3-4 mm in height were prepared using an alloy mold only with light hand pressure. After curing at 37±1°C for 24 hours, some of the pellets were crushed and ground into a fine powder in an agate mortar.

Powdered cement samples were used for subsequent tests where pure Monetite (CaHPO₄) and Hydroxyapatite (HA, Ca₁₀(PO₄)₆(OH)₂) powders were used as controls. For the sake of comparison, pellets of HA prepared according to the method reported in the previous section were also used for all the analyses. Such a method also allowed the comparison of powdered samples versus pelleted samples.
2.3 Results

2.3.1 Setting Time

For measuring setting time, pastes were pressed using light hand pressure between two glass slides to form a flat sample as much as possible. A record of $t_i$ was made and registered as the time in minutes when the light needle did not leave an indentation deeper than 1 mm on the cement surface, and $t_f$ was the time in minutes when the heavy needle failed to leave an indentation deeper than 1 mm on the cement surface. Measurement of the exact setting time is difficult to quantify so the test interval of five minutes was used for sample periods.

After mixing the powder and liquid components together, initial and final setting time were determined and have been written in the table 2.1. Each value is the mean of six measurements.

The liquid to powder ratio (L/P) is a critical factor measuring the setting time. Namely, in beginning trials it was observed that the L/P ratio was directly proportional to the setting time of the cement and lowering this ratio caused an apparent decrease in the setting time of the Monetite cement. It was reported that increasing the L/P ratio also decreased the noticeable viscosity of the paste. Consequently, even though the paste was more workable it required a longer period of time to cure. In most cases, the L/P ratio is chosen depending on the desired characteristics of the MC[103].

In this study, L/P of 0.53 was used. This ratio is the optimum ratio for getting the best setting time and compressive strength. Previously, L/P of 0.75 and 0.4 were tried but the results were not as good as 0.53.
<table>
<thead>
<tr>
<th>Percent of CNT (%)</th>
<th>Initial Setting Time (min)</th>
<th>Final Setting Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8 ± 2</td>
<td>36 ± 4</td>
</tr>
<tr>
<td>0.5</td>
<td>7 ± 3</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>1</td>
<td>6 ± 3</td>
<td>25 ± 5</td>
</tr>
<tr>
<td>2</td>
<td>4 ± 2</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>1 with no surface modification</td>
<td>9 ± 3</td>
<td>39 ± 4</td>
</tr>
</tbody>
</table>

Table 2.1: Setting times of MC-CNT samples with different ratios of CNT

### 2.3.2 Mechanical Properties

Table 2.2 and figure 2-2 to figure 2-4 show the compression strength (MPa) and Young’s Modulus of MC samples versus CNT as reinforcement in ratios of 0, 0.5, 1 and 2%. Each curve is the mean value of the compressive strength of six samples with the error bar which shows the standard deviation (mean ± standard deviation; n = 6). It observed the compressive strength was increased almost twice from 11.09 MPa at 0% CNT to 21.56 MPa at 1% CNT, while the addition of 0.5% and 2% of CNT showed not a comparable improvement to 1% CNT. Young’s modulus also jumped from 350.70 MPa to 526.35 MPa because of adding 1% CNT to the calcium phosphate cement. Another thing which observed in this study it was restriction of strain by adding 1% CNT. As mentioned before, surface modified CNT by producing bonds between MC particles, control the tensile stress. Because of that, it can be seen adding 1% CNT to the paste, decrease strain from almost 15% to 12%.
<table>
<thead>
<tr>
<th>Percent of CNT (%)</th>
<th>Compressive Strength (MPa)</th>
<th>Young’s Modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11.09 ± 1.85</td>
<td>350.70 ± 38.87</td>
</tr>
<tr>
<td>0.5</td>
<td>15.93 ± 1.25</td>
<td>392.92 ± 44.22</td>
</tr>
<tr>
<td>1</td>
<td>21.56 ± 2.47</td>
<td>526.35 ± 62.77</td>
</tr>
<tr>
<td>2</td>
<td>17.56 ± 0.68</td>
<td>315.10 ± 62.09</td>
</tr>
<tr>
<td>1 with no surface modification</td>
<td>10.25 ± 1.65</td>
<td>300.70 ± 35.22</td>
</tr>
</tbody>
</table>

Table 2.2: MC-CNT Mechanical Properties with different ratios of CNT

Figure 2-1: Compressive strength of MC-CNT samples versus different ratios of CNT
Figure 2-2: Young’s modulus of MC-CNT samples versus different ratios of CNT

Figure 2-3: Maximum strain of MC-CNT samples versus different ratios of CNT
2.3.3 Composite Characterization

2.3.3.1 X-Ray Diffraction Analysis

Cement pastes were cured at 37°C in 100% humidity. After this step, they were put outside to dry out. Cured cement samples were crushed and grinded in an agate mortar for three minutes. The fine powder was filled in a quartz sample holder and XRD analysis was conducted.

The XRD pattern of MC-CNT was shown in figure 2-5, both exhibited the characteristic peaks of monetite. In addition, pattern belonging to trace amount of unreacted Ca(OH)$_2$ was also observed. The amount of Ca(OH)$_2$ was estimated to be around 4.4% based on the JADE software analysis.
Figure 2-5: XRD pattern of MC-CNT, 1-Monetite, 2-Ca(OH)$_2$
2.3.3.2 Morphology Observation of Cements

The SEM image presented on Figure 2-6a shows the microstructure of set MC-CNT samples. The structure did not show a specific orientation suggesting a more or less isotropic behavior. The micrograph also illustrated that the cement had some porosity. In higher magnification (figure 2-6b) few CNTs were observed on the surface of the cement. These CNTs were partially interlocked into the crystal structure of formed monetite.

Figure 2-6: SEM pictures of MC-CNT samples (1% wt)
2.3.3.3 Fourier Transform Infrared Spectroscopy Analysis

The FTIR data was shown in Figure 2-7. In the MC-CNT it was showed there are bands of $\text{PO}_4^{3-}$ and $\text{CO}_3^{2-}$. These peaks of carbonate and phosphate showed $\text{NaHCO}_3$ and $\text{H}_3\text{PO}_4$ in the setting solution prepare bonding in the monetite cement.

Figure 2-7: FTIR data of MC-CNT
2.3.4 Cell Culture with Osteoblast Cells

Cell viability was measured after 24 hours and seven days. Statistical sample size (n) was three for all the tests. Three samples of each group were used for after 24 hours, three samples again were used for cell viability on day seven, and one sample of each group was used for SEM micrograph pictures. Samples were put in well plates (Tissue Culture Plate, 12 Well, FALCON®, Becton Dickinson Labware, Franklin Lakes, NJ).

Cell numbers on strips were counted after 24 hours and seven days using CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega). For statistically meaningful results, all experiments were performed in triplicates. Osteoblast attachment on pellets was examined using SEM. Prior to SEM investigations, cells were fixed using 4.5% glutaraldehyde in a cacodylate buffer (pH = 7.4). Osteoblasts were dehydrated through sequential washings in 30%, 50%, 70%, 90%, 95%, and 100% ethanol solutions two times. Samples were then critical point-dried according to previously published techniques and sputter-coated with a thin layer of gold prior to the SEM imaging. [101]
The in vitro tests in figure 2.9 indicate osteoblast cell proliferation on cement samples. After seven days of cell culture, it was observed that the strips with HA had the highest number of osteoblast cells (p<0.05), but the number of osteoblast cells (p<0.05) on MC and MC-CNT samples were close to HA. This is the proof of the MC and MC-CNT biocompatibility.
Figure 2-9: Osteoblast cell numbers for three different compositions
2.4 Discussion

MC-CNT is a paste like material which can be shaped and injected as two necessary factors for filling bone defects. The material is self-setting at 37°C in an incubator with 100% humidity. The mechanism behind the setting of monetite cement is the consumption of free Ca$^{2+}$, HPO$_4^{2-}$, H$_2$PO$_4^{-}$ ions to synthesize monetite crystals. Generally such reactions include the gradual dissolution of Ca(OH)$_2$ in acidic solution and release the cations, which react with the phosphate anions, forming a coordinated network that consolidates into monetite around the unreacted Ca(OH)$_2$. This caused hardening and setting in ceramic body. In all cases, trace Ca(OH)$_2$ was detected on the hardened paste. This phenomenon can be attributed to fact that fast set monetite network formed around unreacted Ca(OH)$_2$ works as barrier to stop further contact of entrapped Ca(OH)$_2$ to acidic environment, hindering the sustained reaction. Adding NaHCO$_3$ buffered the pH of the system to a range where monetite formation is more favored than brushite and monocalcium phosphate monohydrate (MCPM) [104]. The addition of CAM was proposed to improve the handling and injectability of monetite cement [105, 106].

In theory of composites, transferring stress from the filler to the matrix depends on the interfacial bonding between the two phases [107]. CNTs not only have good mechanical properties, but also they can improve mechanical properties of monetite cement. In the case of poor or absent interfacial bonding, CNTs may act as a source of microcracks which leads to failure of the matrix [108]. Additionally, it was reported that regular CNTs have a tendency to agglomerate which causes forming bundles [109], thus resulting in discontinued phase and press concentration sites in monetite matrix. In our
experiment, the incorporation of untreated CNTs, into monetite setting reaction delayed the setting time, decreased the compressive strength, and caused collapse of monetite matrix during compression. On the other hand, surface modified CNTs with the ability to form interfacial bonding to monetite accelerated setting and improving the mechanical performance of the matrix when suitable amount (1 wt%) was applied. However, the uniform dispersion of additives in MC is an unsolved problem even interfacial bonding was induced to additives surface. Extra amount of CNTs still resulted in the discontinued phase in continued monetite body leads to decrease compressive strength. The maximum compressive strength of MC-CNT was double of the cancellous bone. The Young’s modulus of both monetite and MC-CNT matched the value range of cancellous bone [110]. Based on the mechanical testing results, MC-CNT was a good candidate material as cancellous bone substitute.

From composite characterization view, it appears CNTs has no impacts on the crystallite of formed monetite based on the XRD patterns. Additionally, the absorbance spectra of the phosphate region (900-1200 cm\(^{-1}\)) of MC-CNT in FTIR analysis exhibited the presence of several visible peaks and shoulders, confirmed the high crystallite of formed monetite in the presence of surface modified CNTs [111]. The results of biocompatibility test show the proliferation of MC3T3 osteoblast cells on both monetite and MC-CNT (1 wt%). After 7 days cell culture, no significant difference was observed on cells numbers as compared to HA. The SEM images of cell morphology also supported the biocompatibility of monetite and MC-CNTs.
2.5 Conclusion

A novel method of producing MC-CNT (Monetite Cement-Carbon Nanotubes) composite was developed for orthopedic applications in this study which is an important advancement in the orthopedic and dentistry application, as low strength have previously limited the use of MCs in load bearing locations. Surface modified CNTs almost doubled the MC-CNT compressive strength and Young’s modulus in comparison with regular MC. In addition, osteoblast cells attached to the MC-CNT samples as well as HA (Hydroxyapatite) and MC. These attachments of the cells to the surface of the samples showed good biocompatibility of this cement.

Finally, the relative high strength of this cement compare to the cancellous bone, self-hardening, being moldable, injectability, and biocompatibility of this product make it a good filler for bone repair and regeneration.
Chapter 3

Augmentation of Monetite Cement with Surface Modified Chitosan Powder

3.1 Introduction

3.1.1 Chitosan

Chitin and chitosan have been investigated for different applications such as gene delivery, tissue engineering and enzyme immobilization. Chitin is the most plentiful nitrogen-bearing organic compound found in nature and is the second most available polysaccharide next to cellulose[112]. Chitin is a linear polymer arranged by β(1,4)-linked N-acetyl glucosamine (NAG) units which form a three dimensional α-helical pattern stabilized by intramolecular hydrogen bonding[113, 114]. Cuticles of crustaceans particularly shells of shrimp, crab, other byproducts of the food industry, and the exoskeletons of cephalopods are the major sources of chitin production[115]. Chitin is extracted by demineralization and deproteinization of crustacean shells by highly concentrated solutions of sodium hydroxide under the high temperature method.
Figure 3-1: Structure of Chitin and Chitosan. Chitin is composed predominantly of (y) units. Chitosan is composed predominantly of (x) units[116].

Chitosan has broad applications in medical fields, such as wound dressings, hypocholesterolemic agents, blood anticoagulants, antithrombogenics, and drug delivery systems[117]. Uses continue to be found in other fields such as waste-water treatment, food and feed additives, cosmetics, textiles, paper and film technologies[118, 119]. Chitosan is advantageous in the development of microparticles or nanoparticles. This can be used for preparing scaffolds, gene delivery, and drug delivery[117]. Dissolving chitosan in aqueous acidic solutions reduces the need of organic solvents when fabricating particulate systems. In addition, the free amino groups become protonated at low PH levels, which form ionic cross-linking multivalent anions[119]. Furthermore, chitosan is mucoadhesive and has the ability to continue the release of active agents such as the transforming growth factor-β1[120].
Chitosan has considerable potential for tissue regeneration purposes because of its biocompatibility, biodegradability and bioresorbability. Also, chitosan is apt to mold into porous scaffolds with controllable characteristics[121]. Moreover, chitosan surfaces support the attachment and the consequent proliferation of different types of cells, which have been attributed to the high cationic charge density of chitosan [122]. On the other hand, the production of transforming growth factor-beta 1 (TGF-β1), platelet-derived growth factor (PDGF), and maintaining the chondrogenic phenotype are promoted by chitosan[123]. Lahiji et al. [124] and other researchers showed chondrocytes seeded on chitosan film surfaces exhibited and expressed the spherical morphology aggrecans and type II collagens[125]. Chitosan has lots of advantages in bone and cartilage regeneration therapy. For instance, chitosan has been investigated as a support matrix for bones. It can be used as a biocompatible coating for orthopedic and craniofacial implant devices[126, 127]. There is an interest in using chitosan in bone regeneration due to its similarity to GAGs and its ability to interact with common connective tissue components such as collagen to form insoluble complexes.

3.1.2 Using Chitosan in Monetite Cement

Chitosan has a potential to be used in bone regeneration applications. The benefits of using chitosan in Calcium Phosphate Cements (CPCs) as reinforcement phase are known for years. In this study chitosan, although it doesn’t have a good mechanical properties, was used to fill the gaps and cracks between the particles of monetite cement that has not been done yet.
As far as mechanical properties go, MC is a ceramic, and therefore a brittle material. This leads to the creation of a thin bone limiting its usefulness in various stress-bearing locations or unsupported defects[2]. Low strength causes the cement to fail or collapse under low stresses. One of the main purposes of making ceramic composites with reinforcements is to make a material with improved strength and fracture toughness[16]. Poor tensile and compressive strength of CCs as compared with bone makes it inappropriate for load bearing locations[17]. Chitosan as reinforcement phase increases the strength and toughness of DCPAs without counterbalancing bioactivity and biodegradability of the product[3].

3.2 Materials and methods

3.2.1 Cement Powder and Liquid

The cement powder consisted of a mixture of calcium hydroxide, low molecular weight chitosan, water, and setting solution.

3.2.2 Chitosan

Using raw chitosan in MC instead of increasing the mechanical properties, decrease them. An important comment about enhancing bonds between chitosan particles and the cement molecules needs to be mentioned. This issue has been solved by modifying the surface of chitosan with a coating of phosphate to make calcium molecules attach to the cement and makes the cement stronger[128]. Chitosan powder is coated with biomimetic phosphate according to a method modified by Varma et al. [129]. To form the biomimetic calcium phosphate coating, the powder was first phosphorylated to create
functional sites on the surface of the chitosan that facilitate the nucleation and growth of calcium phosphate crystals[130].

In a round-bottomed flask, 3 grams of 98% O-phosphoric acid, 15 grams of urea (CH₄N₂O, Fisher Chemicals, Fairlawn, NJ), 15 mL N,N-dimethyl formamide (C₃H₇NO, Fisher Chemicals, Fairlawn, NJ), and 1 gram of chitosan powder were mixed for three of hours. After finishing this step and getting a completely homogeneous mixture, the blend was heated to 120°C and cooled down for one hour. After cooling, the powder was rinsed and centrifuged three times with deionized water to remove all the extra acid and neutralize the PH. In the study, the mixtures were centrifuged in 50ml centrifuge tubes (Fisher Scientific, Fair Lawn, NJ) for three minutes with the speed of 3000rpm. After centrifuging, the powder left in the centrifuge tube was put in the incubator at 40°C to remove all of the water in the powder. Finally, the powder was ground with an agate mortar and pestle.

3.2.3 Setting Solution

Setting solution for the cement samples was prepared by mixing different reagents and deionized (DI) water. For preparing 15ml of the setting solution, 0.0032gr of citric acid monohydrate (CAM), 6gr of sodium bicarbonate (NaHCO₃), 1.95ml of DI water, and 13.05ml of phosphoric acid (85% H₃PO₄), were mixed respectively as mentioned in the previous chapter.

3.2.4 Cement Synthesis

Cement pastes were prepared by manually mixing calcium hydroxide with setting solution, DI water and surface modified chitosan in an agate mortar by using an agate
pestle. 0.6175 grams of Ca(OH)$_2$ is mixed at least three minutes with 0.8ml of DI water to disperse the Ca(OH)$_2$ completely in the water. The dispersion should be long enough to avoid any agglomeration of calcium hydroxide particles. After the dispersion step, surface modified chitosan powder was added to the sample and again mixed with an agate pestle to completely disperse all of the surface modified chitosan in the solution. The color of the solution after adding surface modified chitosan gradually transitioned from white to light beige.

Finally, 0.75ml of setting solution was added to the materials above and placed in the mold of 12mm diameter and 8mm height to make it a disk sample for compression test and cell culture. Before these tests, the samples were dried in a furnace for four hours and after that they were soaked in DI water for 24 hours before testing to mimic body environment.

3.2.5 Setting Time

After preparing the monetite cement, it was put in an appropriate mold for setting time experiments using the Gillmore needle apparatus. Initial ($t_i$) and final ($t_f$) setting times of cement samples were determined by using Gillmore needle according to the international standard ISO 9917 for dental cement and ASTM C266-89[99, 100]. Gillmore needle was explained in the previous chapter completely.

3.2.6 Mechanical Testing

The purpose of the compression tests was to research the effect of reinforcements with various ratios on mechanical properties and strength of the MC-Chitosan specimens. MC-Chitosan specimens were tested at four different mass ratios of surface modified
Chitosan powder in liquid: 0, 5, and 10. The mixed of MC with surface modified Chitosan powder paste was placed into the circular mold. Cylinder dimensions were carefully measured using a digital caliper. Disk specimens of the composite materials were tested to failure in uniaxial compression test using INSTRON Machine. Compressive load versus compressive extension plots and data were obtained from INSTRON machine too. The crosshead rate of loading was set to 0.5 mm/min. A minimum of six samples were built for each combination of reinforcement. The compressive modulus was determined from the slope of the linear region in the stress-strain diagram[43]. Strength and strain were calculated using the following equations.

\[
\text{(1)}
\]

\[
\text{—— (2)}
\]

\[
\text{—— (3)}
\]

3.2.7 Composite Characterization

3.2.7.1 X-ray Diffraction Analysis

The changes in the crystallographic structures of the Monetite Chitosan produced were examined using X-Ray Diffraction (XRD) using a speed of one degree per minute in continuous scan mode with 40 kV and 44 mA. The XRD data was collected for a 2θ range between 10° and 60°.
3.2.7.2 Morphology Observation of Cements

The morphological developments of the Monetite formed in the presence and absence of surface modified Chitosan particles and soaked either in α-MEM or SBF were monitored using scanning electron microscope (SEM). The samples were first dried out completely and mounted on conducting carbon tape, coated with gold and were visualized with accelerating voltage of 10 KV, magnification of 1.00 K and distance of almost 13 mm.

3.2.8 Cell Culture Experiment

Cell Culture was done on hardened MC and MC-Chitosan samples with MC3T3 cells at 37°C with 100% relative humidity and 5% CO₂ in complete α-MEM. MC3T3 cells were grown on 75 cm² culture flasks at 37°C and 5% CO₂ in complete α-MEM. The culture media was changed every two days until the cells reached a convergence of 90 - 95%, as determined visually with an inverted microscope. The cells were then detached from the surface of the flask using trypsin. The obtained cells were then seeded at a concentration of 10000 cells per well for various assays. MC-Chitosan pellets measuring 12 mm in diameter and 3-4 mm in height were prepared using an alloy mold only with light hand pressure. After curing at 37±1°C for 24 hours, some of the pellets were crushed and ground into a fine powder in an agate mortar.

Powdered cement samples were used for subsequent tests where pure Monetite (CaHPO4) and Hydroxyapatite (HA, Ca₁₀(PO₄)₆(OH)₂) powders were used as controls. For the sake of comparison, pellets of HA prepared according to the method reported in the previous section were also used for all the analyses.
3.3 Results

3.3.1 Setting Time

After mixing the powder and liquid components together, initial and final setting time were determined and have been documented in table 3.1. Each value is the mean of six measurements.

The results signified the increase in setting time can be affected by the amount of chitosan incorporated into the cement composites. This can be explained by the hygroscopic ability of the chitosan gel which causes it to keep water for longer periods of time. Because of that issue, the setting time is affected. The temperature of 60°C was enough to dissolve chitosan in phosphoric acid as reported in Lana et al. [131] paper. This shows heat produced from the reaction between calcium hydroxide and phosphoric acid is able to convert chitosan from powder to gel form. Accordingly, the more chitosan incorporated into the paste, the more time is needed to cure the composite. In previous studies conducted by Wang et al. [132], chitosan considerably increased the setting time of the MC[133].

The liquid to powder ratio (L/P) is a critical factor measuring the setting time. Namely, in beginning trials it was observed that the L/P ratio was directly proportional to the setting time of the cement and lowering this ratio caused an apparent decrease in the setting time of the Monetite Chitosan composite cement. Increasing the L/P ratio also decreased the noticeable viscosity of the paste. Consequently, even though the paste was more workable it required a longer period of time to cure. In most cases, the L/P ratio is
chosen depending on the desired characteristics of the MC[103]. In this study, L/P of 0.53 was used.

<table>
<thead>
<tr>
<th>Percent of surface modified Chitosan (%)</th>
<th>Initial Setting Time (min)</th>
<th>Final Setting Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8 ± 2</td>
<td>36 ± 4</td>
</tr>
<tr>
<td>5</td>
<td>5 ± 2</td>
<td>34 ± 5</td>
</tr>
<tr>
<td>10</td>
<td>17 ± 4</td>
<td>80 ± 11</td>
</tr>
<tr>
<td>5 with no surface modification</td>
<td>14 ± 3</td>
<td>40 ± 4</td>
</tr>
</tbody>
</table>

Table 3.1: Setting times of MC-Chitosan samples with different ratios of Chitosan

3.3.2 Mechanical Properties

Table and figures below show the compression strength (MPa) and Young’s Modulus of MC samples versus surface modified chitosan powder as reinforcement in ratios of 0, 5, 10 and one sample of 5% chitosan with no surface modification. Each curve is the mean value of the compressive strength of six samples with the error bar which shows the standard deviation (mean ± standard deviation; n = 6). It observed the compressive strength was increased almost twice from 11.09 MPa at 0% Chitosan to 23.43 MPa at 5% Chitosan, while the addition of 10% of Chitosan showed not a comparable improvement to 5% Chitosan. Young’s modulus also jumped from 350.70 MPa to 529.75 MPa because of adding 5% Chitosan to the calcium phosphate cement.
Another thing which observed in this study it was restriction of strain by adding 5% Chitosan. As mentioned before, surface modified Chitosan by producing bonds between MC particles, control the tensile stress. Because of that, it can be seen adding 5% Chitosan to the paste, decrease strain from almost 15% to 13%.

Incorporating of chitosan is an important fact in determining the mechanical properties of the Monetite chitosan composites. The data show that the composite cement containing 5 wt% of chitosan has the maximum values of the compressive strength. Other researchers have reported similar results[99]. Similarly, Wang et al. [133] mentioned that the compressive strengths and modulus of MC increased significantly with an increase in chitosan amount. They also documented that if more chitosan was added more than a certain amount which is needed, compressive strength goes down. This indicates that the mechanical strength peaked at a certain percentage of chitosan. Therefore, both the previous paper and this study verify that there is an best possible percentage of chitosan which produces an optimal mechanical strength[133].

The data show that the cement produced without chitosan had the lowest value of the [134]average compressive strength. But, the cement produced with chitosan as a reinforcement phase had higher values. The increase in the compressive strengths in the composites when the chitosan was added is attributed to the presence of the intermolecular forces between the chains of the chitosan and their ability to allocate load under compression[135, 136].
<table>
<thead>
<tr>
<th>Percent of surface modified chitosan (%)</th>
<th>Compressive Strength (MPa)</th>
<th>Young’s Modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11.09 ± 1.85</td>
<td>350.70 ± 38.87</td>
</tr>
<tr>
<td>5</td>
<td>23.43 ± 1.47</td>
<td>529.75 ± 17.90</td>
</tr>
<tr>
<td>10</td>
<td>14.95 ± 3.40</td>
<td>328.24 ± 39.64</td>
</tr>
<tr>
<td>5 with no surface modification</td>
<td>9.23 ± 1.42</td>
<td>292.67 ± 31.58</td>
</tr>
</tbody>
</table>

Table 3.2: MC-Chitosan Mechanical Properties with different ratios of Chitosan
Figure 3-2: Compressive strength of MC-Chitosan samples versus different ratios of Chitosan

Figure 3-3: Young’s modulus of MC-Chitosan samples versus different ratios of Chitosan
Figure 3-4: Maximum strain of MC-Chitosan samples versus different ratios of Chitosan

Figure 3-5: One of the MC-Chitosan samples behavior
3.3.3 Composite Characterization

3.3.3.1 X-Ray Diffraction Analysis

Cement pastes were cured at 37°C in 100% humidity. After this step, they were put outside to dry out. Cured cement samples were crushed and grinded in an agate mortar for two to three minutes. The fine powder was filled in a quartz sample holder and taken to XRD scans.

The XRD pattern of MC-Chitosan was shown in figure 3-6, both exhibited the characteristic peaks of monetite. In addition, pattern belonging to trace amount of unreacted Ca(OH)$_2$ was also observed. The amount of Ca(OH)$_2$ was estimated to be around 3.4% based on the JADE software analysis.
Figure 3-6: pattern of MC-Chitosan, 1-Monetite, 2-Ca(OH)₂
3.3.3.2 Morphology Observation of Cements

The SEM image presented on Figure 3-7 shows the microstructure of set MC-Chitosan samples. The cement was composed of clusters of sharply shaped needle-like monetite crystals. The structure did not show a specific orientation suggesting a more or less isotropic behavior. The micrograph also illustrated that the cement had some porosity. It is also obvious from the SEM picture that surface modified chitosan changed the structure of the monetite cement from plate structure to needle structure.

Figure 3.7- SEM pictures of MC-Chitosan sample (5% wt)
3.3.4 Cell Culture with Osteoblast Cells

Cell viability was measured after 24 hours and seven days. Statistical sample size (n) was three for all the tests. Three samples of each group were used for after 24 hours, three samples again were used for cell viability on day seven, and one sample of each group was used for SEM micrograph pictures. The process of preparing samples for cell culture is the same as what mentioned in the chapter two.

Samples were then critical point-dried according to previously published techniques and sputter-coated with a thin layer of gold prior to the SEM imaging. [101]

Figure 3-8: SEM micrograph of osteoblasts cells (MC3T3) on the surface of the MC-Chitosan sample after seven days
The in vitro tests in figure 3.9 indicate osteoblast cell proliferation on cement samples. After seven days of cell culture, it was observed that the strips with HA had the highest number of osteoblast cells (p<0.05), but the number of osteoblast cells (p<0.05) on MC-Chitosan samples were close to HA. This is the proof of the MC-Chitosan biocompatibility.

![Figure 3-9: Osteoblast cell numbers for three different compositions](image)
3.4 Discussion

MC-Chitosan is a paste like material which can be shaped and injected as two necessary factors for filling bone defects. The material is self-setting at 37°C in an incubator with 100% humidity. The mechanism behind the setting of monetite cement is the consumption of free Ca$^{2+}$, HPO$_4^{2-}$, H$_2$PO$_4^-$ ions to synthesize monetite crystals. Generally such reactions include the gradual dissolution of Ca(OH)$_2$ in acidic solution and release the cations, which react with the phosphate anions, forming a coordinated network that consolidates into monetite around the unreacted Ca(OH)$_2$. This caused hardening and setting in ceramic body. In all cases, trace Ca(OH)$_2$ was detected on the hardened paste. This phenomenon can be attributed to fact that fast set monetite network formed around unreacted Ca(OH)$_2$ works as barrier to stop further contact of entrapped Ca(OH)$_2$ to acidic environment, hindering the sustained reaction. Adding NaHCO$_3$ buffered the pH of the system to a range where monetite formation is more favored than brushite and monocalcium phosphate monohydrate (MCPM) [104]. The addition of CAM was proposed to improve the handling and injectability of monetite cement [105, 106].

In theory of composites, transferring stress from the filler to the matrix depends on the interfacial bonding between the two phases [107]. In the case of poor or absent interfacial bonding, chitosan particles may act as a source of microcracks which leads to failure of the matrix [108]. In our experiment, the incorporation of untreated chitosan, instead of surface modified chitosan, into monetite setting reaction delayed the setting time, decreased the compressive strength, and caused collapse of monetite matrix during compression. On the other hand, chitosan with the ability to form interfacial bonding to
monetite accelerated setting and improving the mechanical performance of the matrix when suitable amount (5 wt%) was applied. However, the uniform dispersion of additives in MC is an unsolved problem even interfacial bonding was induced to additives surface. Extra amount of chitosan still resulted in the discontinued phase in continued monetite body leads to decrease compressive strength. The maximum compressive strength of MC-Chitosan was double of the cancellous bone. The Young’s modulus of both monetite and MC-Chitosan matched the value range of cancellous bone [110]. Based on the mechanical testing results, MC-Chitosan was a good candidate material as cancellous bone substitute.

From composite characterization view, it appears Chitosan has no impacts on the crystallite of formed monetite based on the XRD patterns. On the other hand, in the SEM pictures which took from the MC-Chitosan samples, it was observed that the surface modified chitosan change the crystals of the monetite cement from plate structure to needle structure.

The results of biocompatibility test show the proliferation of MC3T3 osteoblast cells on both monetite and MC-Chitosan (5wt%). After 7 days cell culture, no significant difference was observed on cells numbers as compared to HA. The SEM images of cell morphology also supported the biocompatibility of the MC-Chitosan.
3.5 Conclusion

A novel method of producing MC-Chitosan composite was developed for orthopedic applications in this study which is an important advancement in the orthopedic and dentistry application, as low strength have previously limited the use of MCs in load bearing locations. Surface modified chitosan almost doubled the MC-Chitosan compressive strength and Young’s modulus in comparison with regular MC. In addition, osteoblast cells attached to the MC-Chitosan samples as well as HA and MC. These attachments of the cells to the surface of the samples showed good biocompatibility of this cement.

Finally, the relative high strength of this cement compare to the cancellous bone, self-hardening, being moldable, injectability, and biocompatibility of this product make it a good filler for bone repair and regeneration
Chapter 4

Preparation and Characterization of Monetite Bone Cement from Natural Resources

4.1 Introduction

4.1.1 Egg-shell

In that part of the industry, in which poultry eggs are used in the production of human food or possibly animal feed, disposal of waste products from the eggs, especially in the form of remaining eggshells and egg trays, is an important issue to deal with.

Many egg processing lines handle about 1 million eggs per day[137]. The eggshell constitutes approximately 9 to 10% of the weight of an egg[138]. At an average weight of 65 grams per egg, 1 million eggs produce 5.9 to 6.5 tons of eggshells per day, which is a considerable amount that many facilities have to dispose of every day. Poultry eggshells mainly consist of calcium carbonate with other trace minerals. Organic material, in the form of a protective membrane, is found on the inside of the eggshell. The membrane is usually more strongly attached to the shell and thus remains with the shell during egg-breaking.

Currently, only a few methods of handling eggshells from the egg product industry are employed by the industry. In one case, a screw conveyor carries the
eggshells to a storage facility. From the storage facility the shells are loaded onto a means of transportation. The shells are then driven to a farmer and spread on farmland as a calcium carbonate fertilizer. This procedure is considered the most common way of disposing eggshells throughout the world. Spreading the shells on farmland is comparably uncomplicated but includes transport costs, which are to be covered by the egg product manufacturer. As a rule, the farmer neither pays for nor receives payment for receiving the shells. As a consequence, this solution is an overall expense for the egg product manufacturer.

4.1.2 Using egg-shells in CPC application

The chicken eggshell is 95-97% calcium carbonate (CaCO$_3$) crystals, which are stabilized by a protein matrix[139, 140]. It shows that the eggshells have a good source of calcium which is one of the main components of CPCs. Therefore, one of the ingredient of the cement which contains most amount of calcium can be replaced with other components produce from eggshell.

This work is based on preparation processes of Monetite cement from natural materials that are available in large scale in nature[141, 142]. Monetite is a highly bioactive and biocompatible inorganic material which h is widely used in tissue engineering and bone replacement[4, 103]. In this study, Monetite cement was synthesized using eggshell and setting solution that was added to starting powders at a specific mixing ratio. By replacing calcium source of monetite from calcium product obtained from eggshell, save huge amount of eggshell per day from being dumped.
4.2 Materials and methods

4.2.1 Preparation of powders

For this experiment, left over of raw eggshells have been used. For safety it is better to wash the eggshells before putting them in the furnace. But, it is obvious that all the bacteria and impurities will be cleaned after increasing temperature to more than 800°C. The inside layer, which sticks to the eggshell, is protein. After heat treatment of eggshell, there will not be any protein left.

First of all, all the eggshells were rinsed with water to remove all the egg white from inner layer of eggshells. After that, they were placed in an steel tray which can tolerate high temperature, then put the tray to the furnace to calcinate completely at ranges between 800°C to 1200°C. In the first 30 minutes in the furnace, the color of the eggshell changes from white to black and at the end of three hours they again become white[143]. The final product that is obtained from this process is mostly Calcium Oxide (CaO) and some salts and free ions.

After the powder cooled down, the eggshells were crushed and ground in an agate mortar to get a very fine powder. For synthesizing calcium phosphate powder for eggshells in a big scale and prepare fine powder, ball milling method is preferred. A ball mill, a type of grinder, is a cylindrical device used in grinding or mixing materials like ores, chemicals, ceramic raw materials and paints. Ball mills rotate around a horizontal axis, partially filled with the material to be ground plus the grinding medium. Different materials are used as media, including ceramic balls, flint pebbles and stainless steel balls.
Powder washed with water couple of times because of two reasons. First of all, there should be no free ions and salts in eggshell powder. Secondly, water completed the reaction between Calcium Oxide (CaO) and water which produced Calcium Hydroxide (Ca(OH)$_2$).

\[
CaO + H_2O \rightarrow Ca(OH)_2 \quad (1)
\]

4.2.2 Setting Solution

Setting solution for the cement samples was prepared by mixing different reagents and deionized (DI) water. For preparing 15ml of the setting solution, 0.0032gr of citric acid monohydrate (CAM), 6gr of sodium bicarbonate (NaHCO$_3$), 1.95ml of DI water, and 13.05ml of phosphoric acid (85% $H_3PO_4$), were mixed respectively as explained in the chapter two.

4.2.3 Cement Synthesizing

Cement pastes were prepared by manually mixing the calcium hydroxide obtained by heat treated and washed of eggshell with a specific amount of DI-water in an agate mortar by using an agate pestle. After the complete dispersion of calcium hydroxide in DI-water, setting solution for implementation of the cement was added. The mixing ratios of eggshell powder and the setting solution were decided according to the Ca/P molar ratios to be achieved in the final pastes. Since the goal of this study was to produce $CaHPO_4$-based pastes or putties and to avoid the formation of apatitic calcium phosphates as much as possible, only the Ca/P molar ratio of one was studied.
First of all, 0.6175 gm of eggshell powder is mixed at least five minutes with 0.8ml of DI water to disperse the powder completely in the water. This step should be long enough to disperse all powder in the water. Without ball milling, mixing phase is more critical because particles of Ca(OH)$_2$ obtained from eggshell are coarser in comparison with commercial Ca(OH)$_2$. The dispersion should be long enough to avoid any agglomeration of eggshell particles.

Optimized concentrations of water are important to get the most favorable strength and setting time. Also, by optimizing the amount of water, injectability of the cements by a needle can be improved.

Finally, 0.75ml of setting solution was added to the materials above and placed in the mold of 12mm diameter and 8mm height to make it a disk sample for compression test and cell culture. Before these tests, the samples were dried in a furnace for four hours and after that they were soaked in DI water for 24 hours before testing to mimic body environment.

### 4.2.4 Setting time

After preparing the monetite cement from eggshell powder, it was put in the mold, 12mm diameter and 8mm height, for setting time experiments using the Gillmore needle apparatus. Initial ($t_i$) and final ($t_f$) setting times of cement samples were determined by using Gillmore needle according to the international standard ISO 9917 for dental cement and ASTM C266-89[99, 100]. Gillmore needle was explained in the chapter two completely.
4.2.5 Mechanical Testing

The purpose of the compression tests was to research the mechanical properties and strength of the Monetite cement specimens produced from eggshell. Monetite paste was placed into the circular mold. Cylinder dimensions were carefully measured using a digital caliper. Disk specimens of the composite materials were tested to failure in uniaxial compression test using INSTRON Machine. Compressive load versus compressive extension plots and data were obtained from INSTRON machine too. The crosshead rate of loading was set to 0.5 mm/min. A minimum of six samples were built for each combination of reinforcement. The compressive modulus was determined from the slope of the linear region in the stress-strain diagram[43]. Strength and strain were calculated using the following equations.

\[
\text{(1)}
\]

\[
\text{(2)}
\]

\[
\text{(3)}
\]

4.2.6 Composite Characterization

4.2.6.1 X-Ray Diffraction Analysis

The changes in the crystallographic structures of the Monetite produced from eggshell were examined using X-Ray Diffraction (XRD) using a speed of one degree per minute in continuous scan mode with 40 kV and 44 mA. The XRD data was collected for a 2θ range between 10° and 60°.
4.2.6.2 Morphology Observation of Cements

The morphological developments of the Monetite formed from eggshells and soaked either in α-MEM or SBF were monitored using Scanning Electron Microscopy (SEM). The samples were mounted on conducting carbon tape, coated with gold and were visualized with accelerating voltage of 10 KV, magnification of 1.00 K.

4.2.7 Cell Culture Experiment

Cell Culture was done on hardened monetite cement and Monetite eggshell samples with MC3T3 cells at 37°C with 100% relative humidity and 5% CO₂ in complete α-MEM.

Powdered cement samples used for subsequent tests were pure Monetite (CaHPO₄) and Hydroxyapatite (HA, Ca₁₀(PO₄)₆(OH)₂) powders were used as controls. For the sake of comparison, pellets of HA prepared according to the method reported in the chapter two were also used for all the analyses.

4.3 Results and Discussions

4.3.1 Setting Time

After Mixing the powder and liquid components together, initial and final setting time were determined and have been written in the table 4.1. Each value is the mean of six measurements.
The liquid to powder ratio (L/P) is a critical factor measuring the setting time. Namely, in beginning trials it was observed that the L/P ratio was directly proportional to the setting time of the cement and lowering this ratio caused an apparent decrease in the setting time of the Monetite cement formed from eggshells. It was reported that increasing the L/P ratio also decreased the noticeable viscosity of the paste. Consequently, even though the paste was more workable it required a longer period of time to cure. In most cases, the L/P ratio is chosen depending on the desired characteristics of the CPC[103]. In this study, L/P of 0.53 was used.

<table>
<thead>
<tr>
<th></th>
<th>Initial Setting Time (min)</th>
<th>Final Setting Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monetite produced from eggshell</td>
<td>11 ± 3</td>
<td>37 ± 7</td>
</tr>
<tr>
<td>Monetite Cement</td>
<td>8 ± 2</td>
<td>36 ± 4</td>
</tr>
</tbody>
</table>

Table 4.1: Setting times of MC and Monetite cement produced from eggshell

4.3.2 Mechanical Properties

Table and figures below show the compression strength (MPa) and Young’s Modulus (MPa) of eggshell cement samples. Each curve is the mean value of the compressive strength of six samples with the error bar which shows the standard deviation (mean ± standard deviation; n = 6).
Mean value of compressive strength of the samples was 7.56 MPa with standard deviation of 2.48 MPa. The average of the Young’s modulus of the samples was 337.52 MPa. The maximum amount of strain was 0.28.

<table>
<thead>
<tr>
<th></th>
<th>Compressive Strength (MPa)</th>
<th>Young’s Modulus (Mpa)</th>
<th>Maximum Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monetite produced from eggshell</td>
<td>7.56 ± 2.33</td>
<td>337.52 ± 49.17</td>
<td>28% ± 11.6%</td>
</tr>
<tr>
<td>Monetite</td>
<td>11.09 ± 1.85</td>
<td>350.70 ± 38.87</td>
<td>15% ± 3%</td>
</tr>
</tbody>
</table>

Table 4.2: Mechanical Properties of MC and Monetite cement produced from eggshell
4.3.3 Composite Characterization

4.3.3.1 X-ray Diffraction Analysis

Cement pastes were cured at 37°C in 100% humidity. After this step, they were put outside to dry out. Cured cement samples were crushed and grinded in an agate mortar for two minutes. The fine powder was filled in a quartz sample holder and XRD analysis was conducted.

The XRD pattern of washed and sintered eggshell was shown in figure 4-2, both exhibited the characteristic peaks of Ca(OH)$_2$. In addition, pattern

Figure 4-1: Behavior of one of the Monetite cement samples obtained from eggshell
belonging to trace amount of unreacted CaCO$_3$ was also observed. The amount of CaCO$_3$ was estimated to be around 7.7% based on the JADE software analysis.

The XRD pattern of Monetite cement produced from Eggshell was shown in figure 4-3 both exhibited the characteristic peaks of monetite. In addition, pattern belonging to low amount of unreacted Ca(OH)$_2$ was also observed. The amount of Ca(OH)$_2$ was estimated to be around 3.7% based on the JADE software analysis.
Figure 4-2: XRD pattern of washed and heat treated eggshells powder, 1-Ca(OH)$_2$, 2-CaCO$_3$
Figure 4-3: XRD pattern of Monetite Cement from Eggshell, 1-Monetite, 2-Ca(OH)$_2$
4.3.3.2 Morphology Observation of Cements

The SEM image presented on Figure 4-4 shows the microstructure of set monetite cement samples produced from eggshell. The cement was composed of clusters of sharply shaped plate-like monetite crystals. The structure did not show a specific orientation suggesting a more or less isotropic behavior. The micrograph also illustrated that the cement had some porosity.

Figure 4-4: SEM micrograph of monetite cement produced from eggshell
4.3.4 Cell Culture with Osteoblast Cells

Cell viability was measured after 24 hours and seven days. Statistical sample size (n) was three for all the tests. Three samples of each group were used for after 24 hours, three samples again were used for cell viability on day seven, and one sample of each group was used for SEM micrograph pictures. The process of preparing samples for cell culture is the same as what mentioned in the chapter two.

Samples were then critical point-dried according to previously published techniques and sputter-coated with a thin layer of gold prior to the SEM imaging. [101]

![SEM micrograph of osteoblasts cells (MC3T3) on the surface of the monetite produced from eggshell sample after seven days](image)

Figure 4-5: SEM micrograph of osteoblasts cells (MC3T3) on the surface of the monetite produced from eggshell sample after seven days.

70
The in vitro tests in figure 4-5 indicate osteoblast cell proliferation on cement samples. After seven days of cell culture, it was observed that the strips with HA had the highest number of osteoblast cells (p<0.05), but the number of osteoblast cells (p<0.05) on Monetite cement samples produced from eggshell were close to HA. This is the proof of the biocompatibility of this cement.

![Graph showing osteoblast cell numbers for three different compositions](image)

Figure 4-6: Osteoblast cell numbers for three different compositions
4.4 Discussion

Monetite cement produced from natural resources is a green technology. This method helps saving tons of eggshell which must be dumped every day. This new method protect environment and also help poultry industries to do not have any concerns about the leftover of the eggshells.

Monetite cement produce from eggshell is a paste like material which can be shaped and injected as two necessary factors for filling bone defects. The material is self-setting at 37°C in an incubator with 100% humidity. The mechanism behind the setting of monetite cement is the consumption of free Ca\(^{2+}\), HPO\(_4\)\(^{2-}\), H\(_2\)PO\(_4\)\(^{-}\) ions to synthesize monetite crystals. Generally such reactions include the gradual dissolution of Ca(OH)\(_2\) in acidic solution and release the cations, which react with the phosphate anions, forming a coordinated network that consolidates into monetite around the unreacted Ca(OH)\(_2\). This caused hardening and setting in ceramic body. In all cases, trace Ca(OH)\(_2\) was detected on the hardened paste. This phenomenon can be attributed to fact that fast set monetite network formed around unreacted Ca(OH)\(_2\) works as barrier to stop further contact of entrapped Ca(OH)\(_2\) to acidic environment, hindering the sustained reaction. Adding NaHCO\(_3\) buffered the pH of the system to a range where monetite formation is more favored than brushite and monocalcium phosphate monohydrate (MCPM) [104]. The addition of CAM was proposed to improve the handling and injectability of monetite cement [105, 106].

From composite characterization view, it appears powder obtained from eggshell has Ca(OH)\(_2\) peaks and CPC produced from eggshell formed monetite based on the XRD
patterns. Additionally, the absorbance spectra of the phosphate region (900-1200 cm\(^{-1}\)) of monetite in FTIR analysis exhibited the presence of several visible peaks and shoulders, confirmed the high crystallite of formed monetite.

The results of biocompatibility test show the proliferation of MC3T3 osteoblast cells on both monetite and monetite produced from eggshell. After 7 days cell culture, no significant difference was observed on cells numbers as compared to HA. The SEM images of cell morphology also supported the biocompatibility of monetite and monetite produced from eggshell.

### 4.5 Conclusion

A novel method of producing monetite cement from natural resources was developed for orthopedic applications in this study which is an important advancement in the orthopedic and dentistry application. This monetite cement has some advantages compare to the other CPCs. First of all, it is produced from an inexpensive and recycled material. It means it’s a green biocement. Secondly, mechanical properties of this cement are comparable to monetite cement.

Osteoblast cells attached to the monetite produced from eggshell samples as well as HA and CPC. These attachments of the cells to the surface of the samples showed good biocompatibility of this cement.
Future Works

In future, different reinforcements can be added to the monetite cement to get better mechanical properties. Reinforcement which there is no surface modification is needed. In addition, capability of drug and gene delivery of these cements should be recognized. It is interesting to see that cement can release medicine in the body.

These can be modeled with one of the finite element packages. This helps the engineers to have a better understanding of these cement composites. In parts of body which have complex geometries, modeling by finite element softwares easily give the users stresses and strains in every parts of the cement they want.

Finally, preparing a business plan will be useful for calcium phosphate cements. It will show a clear understanding of the cost and benefits of using bone cements in the body.
References


