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## SYNTHESIS, CHARACTERIZATION AND IN-VITRO BIOACTIVITY OF CaSiO<sub>3</sub>-CaCO<sub>3</sub>-SiO<sub>2</sub> COMPOSITES PRODUCED VIA HYDROTHERMAL REACTION

By

#### BERRA BEYOGLU SIGLAM

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## **ABSTRACT OF THE DISSERTATION**

# Synthesis, Characterization and In-Vitro Bioactivity of CaSiO3-CaCO3-SiO2 Composites Produced Via Hydrothermal Reaction By BERRA BEYOGLU SIGLAM Dissertation Director:

#### **Richard E. Riman**

Given the general increase in human lifespan, there is a growing need for bioactive bone implant material to repair bone defects caused by trauma, infection and tumor. To repair these bone defects, bone implant material should possess biomechanical and biochemical compatibility with natural bone and osteoinductivity to expedite the healing process. The implant material 45S5 bioglass, having great biocompatibility and osteoinductivity properties, gives best biological response. However, it has poor mechanical properties. Therefore, a material is needed that possesses all the required properties. In this study, our overall research goal is to produce CaSiO<sub>3</sub>-based ceramic composites that are mechanically and biologically compatible with human cortical bone and have osteoinductivity comparable to 45S5 bioglass that will promote bone growth and healing. To meet this need, we propose Low Temperature Solidification (LTS, carbonation) method to increase density of High Temperature Sintering (HTS) processed CaSiO<sub>3</sub> that could enhance mechanical properties of CaSiO<sub>3</sub> scaffolds, and control the concentration of Ca and Si ions released from HTS CaSiO<sub>3</sub> to promote biocompatibility and osteoinductivity. For this purpose, we investigated the effect of combining HTS and LTS processes on the microstructure, mechanical properties, the dissolution behavior, in-vitro biocompatibility, and osteoinductivity of CaSiO<sub>3</sub> scaffolds. Processing CaSiO<sub>3</sub> compacts by HTS and LTS methods produced CaSiO<sub>3</sub>-CaCO<sub>3</sub>-SiO<sub>2</sub> composites. XRD patterns indicated development of  $CaCO_3$  phases after carbonation process. Proportional to degree of carbonation, an increase in relative density up to 16 % accompanied by a decrease in porosity, pore size was achieved. Observation of the reaction products filling the pores of CaSiO<sub>3</sub> indicated the compacts effectively densified. The maximum compression strength of 279 MPa and bending strength of 65.5 MPa and fracture toughness of 1.87 MPa.m $^{1/2}$  were achieved with the samples sintered and then hydrothermally reacted. The enhanced relative density and strength and toughness reached by carbonation of green bodies and sintered CaSiO<sub>3</sub> scaffolds improved mechanical compatibility with natural bone, increasing their potential as bone replacement material. The dissolution behavior of processed CaSiO<sub>3</sub> scaffolds were evaluated by Simulated Body Fluid (SBF) immersion. Soluble factor concentrations were found to decrease with increasing degree of carbonation. The drawbacks of rapid dissolution of sintered CaSiO<sub>3</sub> were addressed by carbonation process lowering release of soluble ions. In-vitro cell proliferation and osteogenic differentiation tests were performed to evaluate biocompatibility and osteoinductive potential of processed  $CaSiO_3$  scaffolds, respectively. In-vitro cell experiments showed CaSiO<sub>3</sub> composites produced by carbonation of sintered CaSiO<sub>3</sub> possessed significantly greater proliferation and osteogenic differentiation (p < 0.05) compared to only sintered CaSiO<sub>3</sub> and osteoinductive 45S5 bioglass. Our results suggested that CaSiO<sub>3</sub>-CaCO<sub>3</sub>-SiO<sub>2</sub> composites produced by processing CaSiO<sub>3</sub> ceramics via HTS and LTS methods meet the requirements for repair of bone defects and might be a potential candidate as osteoinductive bone implant material.

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## **Chapter 1: Introduction**

Bioactive materials have a potential to repair bone defects resulting from injury, infection and tumor and speed the healing process. Many researches have been conducted to find a suitable bioactive material for the repair of these bone defects. In the development of new biomaterials for bone replacement, biochemical and biomechanical compatibility of implant material with host bone are essential parameters that must be considered. Moreover, implant materials not only provide structural support (osteoconductivity) but also activate bone regeneration by stimulating osteoblastic cell proliferation and differentiation (osteoinductivity)[1][2] are ideal for the repair of these bone defects. 45S5 bioglass is a well-studied bioactive glass composition having both osteoinductivity and osteoconductivity [3][4][5][6][7]. However, the bioglass has poor mechanical properties, namely high brittleness, low strength and low fracture toughness [8]. Thus, it cannot be used for load bearing applications. The overall research goal of this study is to produce CaSiO<sub>3</sub>-based ceramic composites that are mechanically and biologically compatible with human cortical bone, and have osteoinductivity comparable to 45S5 bioglass in order to promote bone growth and healing processes. In this study, we propose Low Temperature Solidification (LTS) method to increase density of  $CaSiO_3$  ceramics processed by High Temperature Sintering (HTS) which could enhance mechanical properties of CaSiO<sub>3</sub> ceramics cost effectively, and improve biocompatibility and osteoinductivity properties of CaSiO<sub>3</sub> ceramics by suppressing rapid Ca and Si ion release from CaSiO<sub>3</sub> and regulating pH change.

#### **Summary of Contents**

Chapters 2-3 represent two individual papers to be published in a high-ranking journal related to the subject matter. Each of the chapters list collaborators and have separate introductions, experimental procedures, results, discussions, and conclusions. References and figures are found at the end of each relevant chapter. The papers follow an evolution of the thesis work. The first paper investigates microstructural and mechanical behavior of CaSiO<sub>3</sub> scaffolds processed by HTS, or LTS, or their combinations. The second paper evaluates dissolution behavior and in-vitro cell behavior of CaSiO<sub>3</sub> ceramics processed by HTS, or LTS, or their combinations.

Chapter 2 presents an experimental study investigating the effect of combining HTS and LTS processes on the microstructure and mechanical properties of bioactive CaSiO<sub>3</sub> scaffolds. The phase composition, the degree of carbonation, density, porosity, surface area and microstructure of the calcium silicate ceramics after sintering and carbonation were investigated by XRD, TGA, pycnometer, porosimeter, BET, Scanning Electron Microscope (SEM).

Chapter 3 presents experimental work investigating the effect of HTS and LTS processes together on dissolution behavior, and biocompatibility and osteoinductivity properties of CaSiO<sub>3</sub> samples. Dissolution behavior and apatite forming ability of these ceramics were evaluated by immersing processed CaSiO<sub>3</sub> scaffolds into Simulated Body Fluid (SBF). After SBF soaking, ionic concentration of Ca, and Si ions and pH change of SBF and weight loss of the samples were monitored. TF-XRD and FESEM methods were used to investigate apatite formation ability of processed CaSiO<sub>3</sub> scaffolds were evaluated by in-vitro

cytotoxicity, cell adhesion, cell proliferation, and osteogenic differentiation tests respectively.

Chapter 4 summarizes the major conclusions of the previous chapters.

Chapter 5 discusses possible future research directions.

The appendices sections at the end provide more in-depth results from Chapter 2 and 3. Appendix A provides chemical composition of raw CaSiO<sub>3</sub> provided from NYCO. In addition, particle size distribution of raw CaSiO<sub>3</sub> powder was included. Appendix B provides additional FESEM images and EDS spectrums of the CaSiO<sub>3</sub> samples processed by HTS or LTS, or both.

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## **Chapter 2: The Influence of Densification Method on the**

## **Structure and Mechanical Properties of**

## CaSiO<sub>3</sub>-CaCO<sub>3</sub>-SiO<sub>2</sub> Composites

Berra Beyoglu<sup>a</sup>, Richard E. Riman<sup>a</sup>, Malki Pinkas<sup>b</sup>

<sup>a</sup> Department of Material Science and Engineering, Rutgers, The State University of New Jersey, Piscataway, NJ, USA

<sup>b</sup> Negev Nuclear Research Center, Beer Sheva, Israel

*KEYWORDS* Low temperature solidification (*LTS*), carbonation, high temperature sintering (*HTS*), wollastonite, bioactive ceramic, bioactive composite, mechanical properties, strength, fracture toughness

#### Abstract

This study investigates the effect of combining high temperature sintering (HTS) and low temperature solidification (LTS) via carbonation processes on the microstructure and mechanical properties of CaSiO<sub>3</sub> ceramics. The phase composition, the degree of carbonation, density, porosity, surface area and microstructure of the calcium silicate ceramics after sintering and carbonation was investigated by X-Ray Diffraction (XRD), Thermal Gravimetric Analysis (TGA), pycnometer, porosimeter, Brunauer–Emmett–Teller (BET) surface area, Scanning Electron Microscope (SEM). XRD patterns indicated development of CaCO<sub>3</sub> phases after carbonation. By carbonation, 16-20 % increase in relative density, accompanied a decrease in porosity and pore size, and observation of the reaction products filling the pores of CaSiO<sub>3</sub> indicate that the compacts effectively densified. The mechanical properties of the composites were assessed by measuring compression strength, flexural strength, hardness and fracture toughness which used the chevron notched beam method. The compression strength of 279 ±31 MPa and bending strength of 65.5 ±2 MPa, fracture toughness of 1.87 ±0.1 MPa.m<sup>1/2</sup> were achieved with the

samples sintered at 1150°C and then carbonated. This work reveals an enhancement of relative density and strength and toughness by carbonation of green bodies and sintered CaSiO<sub>3</sub> ceramics compared to only sintered CaSiO<sub>3</sub>. Carbonation of green and sintered CaSiO<sub>3</sub> provided the production of CaSiO<sub>3</sub> composites having strength and toughness compatible with human cortical bone using a low cost process. Therefore, CaSiO<sub>3</sub>-CaCO<sub>3</sub>-SiO<sub>2</sub> composites produced by carbonation of green and sintered CaSiO<sub>3</sub> are promising materials for bone replacement applications, especially in load bearing sites.

#### 2.1. Introduction

Calcium silicate bioactive materials including, glasses, glass ceramics and ceramics, are a new generation of biomaterials which participate in the body's effort to repair itself and show biochemical compatibility with natural bone. A common characteristic of these bioactive materials is their release of Si and Ca ions, henceforth referred as "soluble factors". The soluble factors released from the calcium silicate bioactive materials (a) can form a biologically active carbonated apatite layer on the implant surface creating strong bone-implant interfaces and providing structural support for healing, and (b) can enhance cell activity -proliferation [1] and osteogenic differentiation- [2][3] and in-vivo new bone formation in direct contact with material [4]. In this class of materials, 45S5 bioglass has superior biological response. Mechanical properties of 45S5 bioglass, however, mismatch with human cortical bone due to its high brittleness, low mechanical strength (42 MPa in bending/tensile strength) and fracture toughness  $(0.6 \text{ MPa.m}^{1/2})[5][6]$ . Thus, it cannot be used for load bearing applications. This drawback limits its use to very few clinical applications. The crystalline  $CaSiO_3$  ceramics, even though, have better mechanical strength (~50 MPa in bending strength ) and toughness (0.9 MPa.m<sup>1/2</sup>) compared to

bioglasses, their properties should be improved to find application as bone replacement implant. Therefore, the development of bioactive materials that have bone-like mechanical properties –compressive strength of 130-290 MPa, bending strength of 60-150 MPa and fracture toughness of 2-12 MPa.m<sup>1/2</sup> – (Table 1) [7] [8] [9] [10] is crucial.

Bone replacement implants having lower mechanical strength than that of natural bone can fail under mechanical loads. High Temperature Sintering (HTS) is usually an effective process to both densify and strengthen ceramic materials. However, silicate (MSiO<sub>3</sub>) ceramics are difficult to completely densify with HTS because secondary grain growth rapidly encapsulates pores within each grain thereby limiting the density and mechanical strength to low values [11][12]. When the fracture toughness of implant is lower than that of natural bone, once a crack develops in the implant, the crack will propagate faster than that of bone. In general, ceramics are known to be brittle (not tough) materials because of their lack of ductility. In spite of not having an intrinsic toughening mechanism, toughness of these ceramics can be increased to some extent by the addition of reinforcing phases that can contribute to extrinsic toughening mechanisms [13][14][15].

A variety of calcium silicate-based ceramics have been prepared to improve mechanical properties of these implant candidates further by investigating the influence of different processing methods and use of reinforcing secondary phases, as it summarized in Table 2 [16][11][17][18][19][20][1][21][22]. Endo et al (1994), Lin et al (2005), and Carrodeguas et al (2007) studied *conventional high temperature sintering* (HTS) created materials with three-point bending strength of 51 MPa, 95 MPa and compression strength of 48 MPa respectively. Long et al (2006) studied *spark plasma sintering* (SPS) *methods reported* materials with three-point bending strength of 190M Pa, and fracture toughness of 1.67

MPa.m<sup>1/2</sup>. Their other publication reported materials with bending strength of 290 MPa, and fracture toughness of 2 MPa.m<sup>1/2</sup>. Feng P. et al (2014) added hydroxyapatite (HA) as a reinforcing phase to create a material with compressive strength of 27 MPa and fracture toughness of 1.43 MPa.m<sup>1/2</sup>. Ni group (2008) reported CaSiO<sub>3</sub>/ $\beta$ -TCP composites which exhibited a bending strength of 71.7 MPa and a fracture toughness of 0.9 MPa.m<sup>1/2</sup>. Li et al (2015) used the partial substitution of ZnO and  $ZrO_2$  for wollastonite and created material with 36 MPa and 42 MPa three point bending strength respectively [22]. Although some improvements in mechanical properties have been achieved, either not all essential mechanical properties have been presented on those studies or they don't ensure both strength and toughness criteria required for their match with natural bone except one focusing on spark plasma sintering. CaSiO<sub>3</sub> ceramics processed by Spark Plasma Sintering (SPS) reached to relative densities as high as 95 % and possessed promising mechanical properties for bone replacement by suppressing rapid grain growth (with an average grain size of 0.6µm) of material during sintering [20][23][18][19]. However, the high cost of the SPS technique makes the method commercially impractical for bone implant applications. Briefly, the literature shows increase in the density improves mechanical properties of CaSiO<sub>3</sub> ceramics.

Recently, a new densification technique called Low Temperature Solidification (LTS, carbonation) [24] has been reported that could improve strength of calcium silicate based ceramics [24][25]. The method involves a gas passed through the porous matrix conveys reactants to a wetted matrix surface. During LTS process, the rapid gaseous diffusion takes place throughout a porous material preform, and reactive space(s) reacts with wetted porous matrix surface to form a product filling at least a portion of interstitial spaces.

Schematic of LTS process is shown in Figure 1. During LTS process, thermodynamically favorable carbonation reaction shown in Eqn. 1 formed:

$$CaSiO_{3(s)} + CO_{2(g)} = CaCO_{3(s)} + SiO_{2(s)}$$
  $\Delta H^{\circ} = -87 \text{ kJ/mol CO}_2$  (1)

The precipitation of CaCO<sub>3</sub> and SiO<sub>2</sub> inside the compact's pores densifies CaSiO<sub>3</sub> porous compact. Carbonation of CaSiO<sub>3</sub> under relatively low temperature and low pressure ( $<100^{\circ}$ C,  $\sim 2.36$ atm CO<sub>2</sub>) conditions has been demonstrated with this method, resulting in monolithic ceramics with remarkable mechanical properties. The LTS processed CaSiO<sub>3</sub> ceramics have carbonation degree of  $\sim$ % 47.50 and relative density increased to  $\sim$ 81 % from beginning  $\sim$ 64 %. Achieved compressive strength is  $160\pm17$  MPa which is comparable to conventional high temperature sintered CaSiO<sub>3</sub> ceramics [24]. Thus, sintered calcium silicate ceramics with a typical density of 68 % could utilize LTS to increase the density to 85 % under relatively mild conditions

The objective of this study is to determine if LTS can be used on HTS densified CaSiO<sub>3</sub> to increase the density of calcium silicate ceramics as a means to improve its microstructure and mechanical properties. In this case, LTS can be used as a means to further densify porous CaSiO<sub>3</sub> ceramics processed by HTS. Here in this study, we propose LTS method to increase density of HTS CaSiO<sub>3</sub> that could enhance mechanical properties of CaSiO<sub>3</sub> ceramics very cost effectively to reach mechanical properties of cortical bone.

#### **2.2. Material and Method**

#### **Experimental Strategy**

Experiments were performed to understand the effect of High Temperature Sintering (HTS) and Low Temperature Solidification (LTS) processes together on microstructure

and mechanical properties of  $CaSiO_3$  samples. HTS process was used on  $CaSiO_3$  green bodies. Finally, the LTS carbonation process was used on green body and HTS CaSiO<sub>3</sub>. HTS CaSiO<sub>3</sub> and LTS CaSiO<sub>3</sub> were used as control samples. Hereafter, the term HLTS was employed to designate CaSiO<sub>3</sub> ceramics prepared by "high temperature sintering and then low temperature solidification" respectively. Prefixes of 1100, 1150, 1200 accompanied by HTS and HLTS terms are representative of sintering temperatures applied during sintering. Terminology used to define processed CaSiO<sub>3</sub> ceramics are shown in Table 3. Crystalline phase analysis, the degree of carbonation, porosity and pore size distribution, BET specific surface area, SEM were monitored to evaluate physical and chemical characterization of samples after carbonation. Compressive strength, three-point flexural strength, hardness and fracture toughness of ceramic compacts were tested to evaluate mechanical properties of CaSiO<sub>3</sub>. Physical and chemical characteristics of HTS and/or LTS processed CaSiO<sub>3</sub> ceramics and their relation with mechanical properties were assessed. Effectiveness of LTS process on the densification of a porous HTS CaSiO<sub>3</sub> and its contribution into mechanical properties of CaSiO<sub>3</sub> were evaluated.

#### Experimental

#### Raw Material and Preparation of CaSiO<sub>3</sub> compacts

Mineral wollastonite (NYAD<sup>®</sup> 400, NYCO Minerals Inc, Willsboro, NY) with an average particle size of ~9  $\mu$ m was used as the CaSiO<sub>3</sub> source.

The powder compacts of ~13 mm of diameter and ~13 mm of length were prepared by dry pressing using Automatic Carver Press (Model 4532, Wabash, IN) under 148 MPa. The compacts were then dried overnight in the drying oven at 90°C.

#### *High temperature sintering (HTS)*

Powder compacts were sintered in a box furnace (CM Furnaces Inc., Rapid Temp Furnace, Bloomfield, NJ) at 1100°C, 1150°C, and 1200°C respectively for 2 h, with heating rate of 2°C/min and cooling rate of 10°C/min.

#### Low Temperature Solidification (LTS) on Green and Sintered CaSiO<sub>3</sub> Compacts

The green and sintered ceramic compacts were reacted at 90°C with 20 psig CO<sub>2</sub> gas input in a pressure steamer (All American #75x, Wisconsin Aluminum Foundry Co., Manitowoc, WI) for a minimum of 19 h. After the reaction, the samples were dried at 90°C for 24 h.

#### Characterization

X-ray diffraction (XRD) analysis was performed using a Bruker D8 Discover (Bruker AXS Inc., Madison, WI) with  $Cu_{K\alpha}$  radiation ( $\lambda = 1.514$  Å), parallel beam in the range 10-80° (2 $\theta$ ) with a 0.018° step size and 0.5-s dwell time. PDF numbers of 97-020-1537 (wollastonite 1A-CaSiO<sub>3</sub>), 97-008-7716 (pseudowollastonite (high temperature phase of CaSiO<sub>3</sub>)), 97-028-0991 (aragonite-CaCO<sub>3</sub>), 97-001-6710 (calcite-CaCO<sub>3</sub>), 97-001-5879 (vaterite-CaCO<sub>3</sub>), and 98-0039830 (SiO<sub>2</sub>) published by the International Centre for Diffraction Data (ICDD, Newtown Square, PA) was used to identify the phases. Quantitative phase analysis of XRD profiles was refined by Rietveld method using MDI Jade 9 (Materials Data Inc., Livermore, California, USA).

Thermal gravimetric analysis (TGA) was performed on TA Q500 (TA Instruments, New Castle, DE) by heating from room temperature to 1000°C at a rate of 10°C/min under nitrogen atmosphere at a flow rate of 100ml/min. Weight loss in range of 200-800°C [26]

corresponds to weight percentage (wt %) of  $CO_2$  released as a result of carbonate decomposition as shown in Eqn. 2:

$$CaCO_{3(s)} = CaO_{(s)} + CO_{2(g)} \uparrow$$
<sup>(2)</sup>

The weight percentage of CO<sub>2</sub> ( $m_{CO_2}$ %) is proportional to the degree of carbonation ( $\lambda$  <sub>TGA</sub>(%)) as shown in Eqn. 3:

$$\lambda_{\text{TGA}} (mol \%) = (m_{\text{CO}_2}\%) \times \left(\frac{M_{CaSiO_3}}{M_{CO_2}}\right)$$
 (3)

$$\lambda_{\text{TGA}} (mol \%) = \left(1 - \left(\frac{(m_i - m_{CO2})}{(m_i)}\right)\right) \times \left(\frac{M_{CaSiO_3}}{M_{CO_2}}\right)$$

 $m_i = initial \ sample \ mass \ in \ grams$  $m_{CO_2} = mass \ of \ CO_2 \ in \ grams \ released \ from \ sample$  $M_{CasiO_3} = molecular \ weight \ of \ CaSiO_3, 116.16 \ g/mole$  $M_{CO_2} = molecular \ weight \ of \ CO_2, 44.01 \ g/mole$ 

The net weight gain after carbonation process corresponded to the  $CO_2$  intake during the carbonation reaction. The mass gain of each sample set was recorded after carbonation. The degree of carbonation ( $\lambda_w$ (%)) was calculated from net weight gain as follows:

$$\lambda_{\rm w}({\rm mol}\ \%) = \frac{(m_f - m_i) \times (M_{CaSiO_3})}{(m_i) \times (M_{CO_2})} \times 100$$
(4)

 $m_i = initial \ sample \ mass \ in \ grams$  $m_f = final \ sample \ mass \ in \ grams \ (after \ carbonation)$  $M_{CaSiO_3} = molecular \ weight \ of \ CaSiO_3, \ 116.16 \ g/mole$  $M_{CO_2} = molecular \ weight \ of \ CO_2, \ 44.01 \ g/mole$  *Calcimetry* (Eijkelkamp, ART No 08.53, Agrisearch Equipment, Morrisville, NC) was also used to determine degree of carbonation ( $\lambda_c$ ) volumetrically [26]. The sample was placed in a flask bottle connected to a burette and the beginning water level was recorded. When hydrochloric acid were added to the sample, the carbonates available in the sample convert into CO<sub>2</sub> gas leading to rise in water level. Carbonate percentage was computed by measuring the volume of carbon dioxide evolved during the reaction of samples with hydrochloric acid. Degree of carbonation ( $\lambda_c$ ) can be calculated from Eqn. 5:

$$n_{re-CaSiO3} = n_{CaCO3} = \frac{m_{CaCO3}}{M_{CaCO3}}$$

$$n_{un-CaSiO3} = \left[\frac{(100 - (n_{CaCO3} * M_{CaCO3}) - (n_{SiO2} \times M_{SiO2}))}{M_{CaSiO3}}\right]$$

$$\lambda_{\rm C}(\text{mol }\%) = \frac{(n_{\rm re-CaSiO3})}{(n_{un-CaSiO3}) + (n_{\rm re-CaSiO3})} \times 100$$
(5)

 $n_{re-CaSiO3} = mole of reacted CaSiO_3$   $n_{un-CaSiO3} = mole of unreacted CaSiO_3$   $n_{CaCO3} = mole of CO_2 released from the sample$   $n_{SiO2} = mole of SiO_2$ M = moleculer weight

*Relative density* ( $\rho_r$ ) was calculated from measured *bulk density* ( $\rho_b$ ) with respect to *theoretical density* ( $\rho_{th}$ ) as follows:

$$\rho_{r} = \left(\frac{\rho_b}{\rho_{th}}\right) \tag{6}$$

*Bulk density* was measured by mercury porosimeter (Autopore IV, 9400, Micromeritics Instrument Corp., Nocross, GA). *Apparent density* ( $\rho_a$ ) of the compacts was measured by

helium pycnometer (Accupyc-1430, Micromeritics Instrument Corp., Nocross, GA). *Theoretical density* ( $\rho_{th}$ ) of composites was calculated from rule of the mixtures, using volume percent (v) of each phase calculated from their degree of carbonation as follows:

$$\rho_{th} = (v_{caco3} \times \rho_{caco3}) + (v_{casio3} \times \rho_{casio3}) + (v_{sio2} \times \rho_{sio2})$$
(7)

*Here,* v is volume percent and  $\rho$  is theoretical density of corresponding phase in the composite. Theoretical density of CaCO<sub>3</sub> phases formed by carbonation was calculated from Rietveld analysis data using volume percent of each CaCO<sub>3</sub> phase and their theoretical densities.

*Theoretical density* ( $\rho_{th}$ ) (true density) was also directly measured by pycnometer method after the sample was ground until there is no more increase in density with smaller particle size, so closed pores were eliminated [27][28].

*Porosity* and *pore size distribution* of the ceramic compacts were measured by Mercury (Hg) Intrusion Porosimetry (MIP, AutoPore IV 9400, Micromeritics Instrument Corp., Norcross, GA). Prior the measurement, the samples were oven-dried overnight at 90°C.

*The specific surface area* of the samples was measured using the Brunauer–Emmett–Teller (BET) method (TriStar II 3020, Micromeritics Corp., Norcross, GA). Prior the measurement, the samples were degassed in a degasser (VacPrep061, Micromeritics Corp., Norcross, GA) under flowing nitrogen gas overnight at 300 °C.

Sample *microstructure* of ion beam milled sections were observed by field emission scanning electron microscopy (FESEM) using a Zeiss Sigma FESEM (Carl Zeiss, Oberkochen, Germany) at an accelerating voltage of 15 kV and working distance of 8.5 m. Sample surfaces were cross-section milled (C5) for 5 h and subsequent flat milled for 5

min at 6 kV accelerating voltage using an ion beam milling system (Hitachi IM4000, Hitachi High-Technologies Corp., Tokyo, Japan). Sample surfaces were sputter coated with 30 nm thick gold coatings (Electron Microscopy Sciences, model# EMS 150T ES, PA). Energy Dispersive Spectroscopy (EDS) was also used to measure chemical composition.

#### **Mechanical Properties**

*Compressive strength, three-point flexural strength* and *fracture toughness* of ceramic compacts were measured by a mechanical testing machine (Model 4505, Instron Corp., Canton, MA). *Compressive strength measurements* were performed using 100 kN load cell equipped test frame at a crosshead speed of 0.5 mm/min on cylindrical samples with ~13 mm in height and ~13 mm in diameter. A set of 6 samples was used to calculate mean compressive strength and its standard deviation (shown in parenthesis). *The elastic modulus* for each sample was determined from the slope of a linear portion of the stress vs strain plot of compressive test data.

*Flexural Strength* was obtained in a three-point bending with 40 mm span size and using 1 kN load cell equipped test frame, at a crosshead speed of 0.5 mm/min on samples with dimensions of 6 mm x 3 mm x 45 mm. Flexural strength was calculated using the Eqn.8:

$$\sigma_{3pb} = \frac{_{3PL}}{_{2BD^2}} \tag{8}$$

Where P is break force, L is span size, B is width, and D is thickness of a specimen. An average of 6 samples was used to calculate the average strength and its standard deviation (shown in parenthesis). *Hardness* was measured by Vickers micro-hardness indentations (VHN, M-400-G3, LECO Corporation, St. Joseph, MI) by applying a load of 9.8 N for 10

s on polished samples (ASTM 1327). After indentation, indentation diameters were measured using an optical microscopy (Keyence Corp, VHX-5000, Itasca, IL). Hardness was calculated according to Eqn. 9

$$H = 0.0018544 \left(\frac{P}{d^2}\right) \tag{9}$$

Here, P is force (N) and d is the average indentation diagonal (mm).

*The fracture toughness* of ceramic compacts was measured by chevron notch flexure method following the ASTM C-1421 standard [29]. Since both mechanical strength and toughness are the fundamental properties for bone replacement materials, sample sets meet a compressive strength criteria of 130MPa and flexural strength criteria of 50 MPa or higher were selected for fractural toughness evaluation. Test specimen and 3-point fixture configurations are shown Fig 11(a). Sample dimensions were 6.35 mm × 6.35 mm × 45 mm which corresponds to the geometry B in the standard. An average of 8 samples was used for fracture toughness measurements. The notches were cut on using a diamond blade with a thickness of 0.15 mm using a custom designed fixture. Chevron-notched beams were fractured in a three point flexural test fixture (40 mm span) with a crosshead rate of 0.025 mm/min. The chevron notch fracture toughness ( $K_{Ivb}$ ) was calculated using Eqn.10:

$$K_{Ivb} = Y_{min}^* \left[ \frac{P_{max} S_0 \, 10^{-6}}{BW^{3/2}} \right]$$
(10)

Where  $Y^*_{min}$  is the minimum stress intensity factor,  $P_{max}$  is maximum load (N) that occurs during stable crack propagation;  $S_0$  is the outer support span (mm); B is the specimen width (mm); and W is the specimen thickness (mm). Load-time curves were used to evaluate stable crack growth. Post-test measurements were performed using optical microscopy (Keyence Corp, VHX-5000, Itasca, IL) at 30X magnification. The crack surfaces and microstructure sections perpendicular to crack surface were observed using electron microscopy (FESEM) (Carl Zeiss, Oberkochen, Germany) to define fracture mode and crack propagation paths in these ceramics respectively. Microstructure sections were ground using SiC sand paper with grit sizes of 600, 800, and 1200. Sample surfaces were sputter coated with 30 nm thick gold coatings (Electron Microscopy Sciences, model# EMS 150T ES, Hatfield, PA).

#### 2.3. Results

The phase composition of raw CaSiO<sub>3</sub>, HTS, LTS, and HLTS samples was identified using x-ray diffraction (Fig. 1). Before carbonation, wollastonite (a low temperature phase of CaSiO<sub>3</sub>) was observed for raw and sintered samples at 1100 and 1150°C. Pseudowollastonite (PsW, high temperature phase of  $CaSiO_3$ ) was the only detected primary phase for samples sintered at 1200°C, as a result of phase transformation from wollastonite to pseudowollastonite known to occur usually at temperatures of 1125±10 °C [30]. After carbonation, all emerging peaks corresponded to reaction product  $CaCO_3$ phases; calcite, aragonite and vaterite. Table 4 summaries the quantitative phase analysis by Rietveld refinement. The primary CaCO<sub>3</sub> polymorphs formed during carbonation were (a) 'calcite' and "aragonite" for only reacted samples, (b) "calcite" and metastable "vaterite" for sintered and then reacted samples. Vaterite is the least stable polymorph of CaCO<sub>3</sub>; calcite is the most stable one [31]. During the reaction, the initial formation of vaterite was expected to convert into aragonite or calcite. Andreassen (2005) reported if vaterite is exposed to water, it can transform to more stable aragonite or calcite [32] [33]. Therefore, the formation of different CaCO<sub>3</sub> phases could arise from the presence of hydroxyl groups originally present in raw CaSiO<sub>3</sub> and disappear in course of sintering.

While vaterite phase present with calcite for sintered samples, aragonite takes the place of vaterite in the reacted green CaSiO<sub>3</sub>. Thus, it can be concluded that during the carbonation the presence of hydroxyl groups in the CaSiO<sub>3</sub> is one of the factors affecting the form of reaction products. After reaction, there was no a rising SiO<sub>2</sub> peak detected. It can arise from its presence in non-crystalline form. Increasing sintering temperature decreased the intensity of carbonate peaks. It can be concluded that the degree of carbonation decreased with sintering temperature as a result of decrease in surface area of material that the carbonation reaction can generate.

Thermal gravimetric analysis (TGA) curve of CaSiO<sub>3</sub> before and after carbonation reaction is shown in Figure 2 (a). Weight loss observed up to 200°C correlated with removal of physically adsorbed water while weight loss between 200-800°C corresponded to decomposition of CaCO<sub>3</sub> [26]. According to the plot, HTS samples showed no weight loss in this range, indicating there was no calcium carbonate prior to the reaction. Thermal analysis confirmed the presence of carbonate phase after reaction. The extent of carbonation of the reacted samples was maximum for carbonated green CaSiO<sub>3</sub> (LTS) and decreasing with increasing sintering temperature for HLTS samples. Degree of carbonation as molar CaCO<sub>3</sub> percentage present in the HTS, LTS, and HLTS samples was measured by TGA, net weight gain, and calcimeter methods as shown in Fig 2(b) and Table 5. For reacted samples, while degree of carbonation for green CaSiO<sub>3</sub> (LTS) was 52 % and it decreased to 46, 36, and 12 % with increasing sintering temperature for pre-sintered samples. Decrease in the degree of reaction with increase in sintering temperature can be correlated with a reduction in surface area of CaSiO<sub>3</sub> compacts that reactant gas interacts. Due to consistency of data collected from TGA, net weight change, and calcimeter, it can

be concluded that the mass gain observed after reaction arose from the carbonation reaction.

Densification of HTS, LTS, and HLTS samples is shown in Fig 3. Our relative density data of HTS samples showed conventional high temperature sintering has limited contribution to densification of 65 % dense green body of CaSiO<sub>3</sub> with only a ~% 3 increase in relative density. Therefore, HTS is an inefficient method to densify CaSiO<sub>3</sub>. Our data is consistent with what is reported in literature [16] [11]. When carbonation applied on green and sintered CaSiO<sub>3</sub>, relative density increased up to 85 %. Degree of reaction was the main reason for a steep increase in relative density. In addition, in comparison to green CaSiO<sub>3</sub> the reaction percentage was decreasing (from 52.7 to 37 %) with increasing sintering temperature, however relative density decrease was negligible for LTS, H1100LTS and H1150LTS samples with values ranging from 84.85 to 83.62 %. There should be other factors contributing to increase relative density.

The pore size distribution of HTS, LTS and HLTS samples is shown in Fig 4(a) and Table 6. For HTS samples, the pore size increased from 0.6 to 2.7  $\mu$ m with increasing sintering temperature ranging from 1100 to 1200°C. After carbonation of green CaSiO<sub>3</sub> (LTS), the pore size decreased substantially to 0.025-0.034  $\mu$ m. For HLTS samples, the pore size distribution decreased proportionate to degree of carbonation of sintered CaSiO<sub>3</sub>. The pore size distribution was 0.0094-0.057  $\mu$ m for H1100LTS samples, 0.187-0.246  $\mu$ m for H1150LTS samples, and 1.655-1.023  $\mu$ m for H1200LTS samples. Porosity change is shown in Fig 4 (b). For HTS samples, porosity decreased from 34 to 31 % with increasing sintering temperature. The pore size increase with a negligible porosity decrease indicated grain growth was active during sintering. Porosity data also proved CaSiO<sub>3</sub> system did not

densify effectively by HTS. The porosity of LTS samples decreased to 14.6 %. For HLTS samples, the porosity reduced to 15.35-27.33 % depending on pre-sintering temperature and the degree of carbonation. By carbonation, a decrease in pore volume and pore size indicated reaction products filled the pores and narrowed the pore size. Carbonation of green CaSiO<sub>3</sub> (LTS) decreased the pore volume and pore size narrowed down to mesopores (2-50 nm). For sintered and then carbonated (HLTS) samples, the pore size and pore volume decreased, compared to HTS samples, proportional to their degree of carbonation. Porosimetry data confirmed the densification of samples following carbonation process.

BET surface area of HTS, LTS, and HLTS samples are shown in Fig 5. The surface area of raw CaSiO<sub>3</sub> powder measured as 1.91  $m^2/g$ . For HTS samples, the surface area decreased with increasing sintering temperature (0.93, 0.40, 0.14  $m^2/g$ ). A reduction in surface area of HTS samples may arise from coarsening of particle and pore surfaces and increase in grain and pore size. Surface area of LTS samples was 0.94  $m^2/g$ . The surface area of HLTS samples increased -2.10 and 0.75 and 0.38 m<sup>2</sup>/g, respectively, with increasing sintering temperature- in comparison to prior-carbonation values (HTS samples). Increase in the surface area were proportional to their degree of carbonation. The surface area change after carbonation might be arisen from formation of reaction products -CaCO<sub>3</sub> and silica rich leached layer- and a decrease in the pore size and the porosity. Reaction products may tend to increase the surface area due developed small CaCO<sub>3</sub> phase particles and formed leached layers. Decrease in final pore size and volume as a result of accumulation of reaction products on the surface of pore walls throughout pore network should decrease the surface area. BET data also indicates the degree of carbonation was proportional with surface area of the starting material. For conditions CaSiO<sub>3</sub> sintered before carbonation, a reduction in the reaction percentage can be correlated with decrease in surface area of  $CaSiO_3$  by increasing sintering temperature. During carbonation, the layer of reaction products,  $CaCO_3$  and silica rich amorphous phase, surrounding core  $CaSiO_3$  phase became thicker as the reaction continued and slowed down further carbonation reaction due to limited the diffusion of Ca ions through silica rich diffuse layer [34]. This finding indicates surface area is an important parameter affecting degree of reaction during carbonation.

Back Scattered Electron (BSE) images collected from HTS, LTS and HLTS samples are shown in Fig 6 (a-b). Both mesopores and macropores (black regions) having consistent pore size with porosimeter were observed in these micrographs. For HTS samples (Fig 6(a)), when sintering temperature was increased to 1150°C, the morphology changed to continuous network of solid  $CaSiO_3$  (grey) and porosity (black) while the acicular nature of wollastonite mineral was still maintained. With further increase in sintering temperature to 1200°C, grain and pore growth shows coarsening (rapid grain growth) mechanism became predominant for  $CaSiO_3$  system and the particle morphology changed to honeycomb-like structure. For LTS samples, pore filling reaction products were clearly detected. The core shell structure produced by LTS including cores of partially carbonated acicular CaSiO<sub>3</sub> grains surrounded by SiO<sub>2</sub>-rich layers that are in turn encapsulated by CaCO3 particles were observed. For HLTS samples, the same core-shell structure were observed and with increasing sintering temperature from 1100 to 1150°C, a microstructure having more pronounced phase boundaries appeared. Each phase having a different tone of gray due to difference in their average atomic number can be identified: (a) brightest regions were unreacted CaSiO<sub>3</sub> cores, (b) surrounded by a dark gray SiO<sub>2</sub> rich amorphous phase, (c) surrounded by CaCO<sub>3</sub> phase in medium gray tone, and (d) black regions were pores. EDS chemical maps also confirmed this core shell structure and arrangement of each compound after reaction. After carbonation, the presence of reaction products filling the pores of CaSiO<sub>3</sub> was proportional with degree of reaction. Carbonation of sintered samples created core shell structures having more discrete phase boundaries due to sintering of small CaSiO<sub>3</sub> particles to big particles during heat treatment. According to EDS maps of carbonated samples, in addition to CaCO<sub>3</sub> phase regions, SiO<sub>2</sub> rich regions can also be clearly observed in the microstructure, even though they cannot be identified using X-ray diffraction. Therefore, we conclude that the formed silica-rich layer is an amorphous phase.

#### **Mechanical Properties**

Fig 7 shows (a) characteristic stress versus strain plots collected during compression tests and (b) comparison of the strength for HTS, LTS and HLTS samples. During strength tests, samples showed brittle behavior with a steep decline in stress after ultimate strength was reached. HTS samples showed a gradual increase in the average strength from 41.5 to 129.7 MPa with increasing sintering temperature from 1100 to 1150°C. However, the strength decreased to 112.9 MPa with further increase in the sintering temperature to 1200°C showing sintering at that temperature fails contribute additional strength. Average strength of carbonated green CaSiO<sub>3</sub> (LTS) was measured as 276.7 MPa. For the HLTS samples, the average strength was recorded as 253.4, 279.2 and 120 MPa for sintering temperatures of 1100, 1150 and 1200°C, respectively. A great decrease in the mechanical strengths of CaSiO<sub>3</sub> compacts sintered at 1200°C and then reacted (H1200LTS) can be attributed to the abnormal grain growth [35] and negligible contribution from carbonation process due to huge decrease in the surface area of pre-sintered samples. Fig 7 (c) shows compression strength change depending on relative density of the processed samples. As expected, compression strength increased with increasing relative density.

The average *elastic moduli* of compression samples is shown in Fig 8. For HTS samples, the modulus increased from  $2.3\pm0.2$  GPa to  $17.7\pm0.4$  GPa with increasing sintering temperature. The modulus of carbonated samples increased to  $20.7\pm2.5$  GPa and decreased with increased sintering temperature to  $18.7\pm1.5$  GPa. An increase was expected in the elasticity modulus which varied linearly with decreasing porosity and increasing sintering temperature [36]. Therefore, increase in the moduli by HLTS can be attributed to densification (decrease in porosity) by sintering and introduction of reaction products into the pores of CaSiO<sub>3</sub> compacts. Elastic moduli of processed CaSiO<sub>3</sub> samples are compatible with the moduli of cortical bone which is in the range of 3-30 GPa.

*Flexural strength* of HTS, LTS, and HLTS samples showed a similar trend with compressive strength data (Fig 9). By applying HTS, achieved maximum bending strength was  $47.2\pm2.5$  MPa for sintering temperature of  $1150^{\circ}$ C. The strength increased to  $70.0\pm2.6$  MPa by carbonation of green CaSiO<sub>3</sub> (LTS) and  $65.5\pm2.1$  MPa by carbonation of sintered CaSiO<sub>3</sub> (HLTS). Both compressive and flexural strength of the carbonated samples surpassed those of the only sintered CaSiO<sub>3</sub> (HTS) samples.

*Vickers hardness* of HTS, LTS, and HLTS samples is shown in Fig 10. With increasing temperature of HTS samples, hardness increased from 0.3 to 0.79 GPa. Hardness of LTS samples was 2.32 GPa, while hardness of HLTS samples was in the range of 1.98-0.75 GPa showing a decrease with decreasing degree of carbonation. The increase in hardness was proportional with a decrease in porosity as a result of sintering and carbonation reaction.

In addition, reaching highest hardness with carbonated green CaSiO<sub>3</sub> was expected due to formation of high density aragonite phase instead of lower density vaterite.

*Fracture Toughness* of processed CaSiO<sub>3</sub> ceramics is shown in Fig 11. A load-time plot (Fig 11(b)) shows that the validity requirement of stable crack extension was met (This test verifies that slow crack growth behavior was evident). Fig 11 (c) shows a comparison of toughness values of HTS, LTS, and HLTS samples. Whereas the fracture toughness of HTS1150 samples (CaSiO<sub>3</sub> sintered at 1150°C) was  $0.96\pm0.05$  Mpa $\sqrt{m}$ , H1150LTS samples (CaSiO<sub>3</sub> sintered at 1150°C and then carbonated) reached the maximum toughness value of  $1.87\pm0.13$  Mpa $\sqrt{m}$ . LTS samples also reached to similarly high toughness  $(1.76\pm0.2 \text{ Mpa}\sqrt{\text{m}})$ . The fracture toughness by carbonation of green and sintered CaSiO<sub>3</sub> ceramics was close to that of cortical bone and about two times of the conventionally sintered CaSiO<sub>3</sub> recorded in this study and also reported previously [1]. Fig 11 (d) shows fracture surfaces with microstructure sections perpendicular to fracture surface (in direction of crack propagation) of HTS and HLTS samples after the test. As expected from ceramic materials, samples showed characteristic brittle fracture behavior, by failing a catastrophically after a small amount of elastic strain [15]. As it can be seen from SEM micrographs, while fracture mode of CaSiO<sub>3</sub> was cleavage (transgranular), CaCO<sub>3</sub> showed intergranular fracture. After carbonation of green (LTS) and sintered CaSiO<sub>3</sub> (HLTS), an increase in fracture toughness can be associated with crack deflection in presence of porefilling CaCO<sub>3</sub> particles which show intergranular fracture behavior. Table 7 summarizes the effect of HTS, LTS, and HLTS processes on mechanical properties of CaSiO<sub>3</sub>.

#### 2.4. Discussion

Experimental results of this study support our proposed hypothesis that carbonation process improves the densification of sintered CaSiO<sub>3</sub> ceramics and so enhances mechanical properties of CaSiO<sub>3</sub> ceramics cost effectively and reached to mechanical properties similar to those of cortical bone.

Carbonation of (a) green CaSiO<sub>3</sub> (LTS), (b) CaSiO<sub>3</sub> sintered at 1100°C (H1100LTS) and (c) CaSiO<sub>3</sub> sintered at 11500°C (H11500LTS) yielded similarly enhanced densification and mechanical strength and toughness. Actually, HLTS samples possessed a relatively lower degree of carbonation compared to LTS samples (52.7%) and their degree of carbonation decreased from 45 to 12.6 % with increasing sintering temperature of CaSiO<sub>3</sub>. The reduction in the degree of carbonation can be explained by a decrease in the surface area of  $CaSiO_3$  by increasing sintering temperature that reaction gas can interact which surface of  $CaSiO_3$  to produce reaction products. As a result of lower degree of carbonation, it should have been expected HLTS samples have lower densification and less enhancement in mechanical properties compared to LTS samples. However, the relative density of HLTS CaSiO<sub>3</sub> reached to relative density of LTS CaSiO<sub>3</sub> and thus both LTS and HLTS samples reached to high strength values. This finding points to other mechanisms that should contribute to densification of HLTS samples to reach similar densification and mechanical properties with LTS CaSiO<sub>3</sub>. Our data showed relative density of HLTS CaSiO<sub>3</sub> increased by two other factors: (a) the variation in the polymorphic form of carbonate phases formed during the reaction. After carbonation, while aragonite and calcite phases formed on green CaSiO<sub>3</sub>, vaterite takes the place of aragonite during the carbonation of sintered CaSiO<sub>3</sub>. Since vaterite has lower theoretical density (2.645g/cm<sup>3</sup>) than that of aragonite (2.95 g/cm<sup>3</sup>), when vaterite is formed instead of aragonite, the relative density of the composite increased around 2 %. For example, H1150LTS samples have relative density of 83.6 %. However if aragonite forms on those samples as it's in LTS samples, the relative density of the composite would be 81.4%. Furthermore, (b) high temperature sintering process contributes to densification of CaSiO<sub>3</sub> even though it is not an effective way to fully densify CaSiO<sub>3</sub>. Especially at sintering temperature of 1150°C which is the highest temperature before coarsening initiates, sintering process increased the relative density around ~3 %. Briefly, our data showed even though there was a decrease in the degree of reaction of HLTS samples compared to LTS samples, expected relative density difference (~% 5) arose from lower degree of carbonation of HLTS samples compensated by HTS process and formation of vaterite polymorph. Due to their similar relative densities, similarly high mechanical strengths were reached with LTS, H1100LTS and H1150LTS samples.

The improvement in fracture toughness was reached by all carbonated samples, LTS, H1100LTS, and H1150LTS samples compared to the HTS sample. It can be interpreted that carbonation reaction might create toughening phases that can activate extrinsic toughening mechanisms in the material structure. Perhaps the pore-filling CaCO<sub>3</sub> phase has crack energy absorbing properties. The 95 % improvement of fracture toughness by carbonation can be due to crack deflection by carbonation reaction products.

Dependent on pre-sintering temperature before carbonation, differences in the pore sizes and microstructure were detected. Out of LTS, H110LTS and H1150LTS samples, all reached to similarly improved densification and fulfilled mechanical compatibility requirements, H1150LTS samples having relatively bigger pore size and more uniform phase distribution in the microstructure can be more promising material for biomedical

applications. Big pores are better for tissue growth and vascularization. In addition to those, variation in their response in biological medium is also expected due to differences in their degree of carbonation and the solubility of reaction products. Future work will focus on the relationship of the carbonation and the in-vitro bioactivity behavior of HLTS samples. Moreover, CaSiO<sub>3</sub> composites produced by carbonation of sintered CaSiO<sub>3</sub> in this study has advantageous over SPS densified CaSiO<sub>3</sub> from few aspects considering their use for biomedical applications. First, grain size is around 0.6  $\mu$ m after SPS, while the grain size is in the range of 5-15  $\mu$ m after carbonation process. Nano scale materials are not promising biomedical applications due to their higher surface area potentially have higher dissolution rate. Second, considering both SPS and carbonation processes produced materials having mechanical properties compatible with cortical bone, material having higher porosity and bigger pore size preferred over denser material and small pore size. Third, high cost and shape limited production via SPS techniques make it less promising method for production of variety of shaped bone substitutes. In contrast, carbonation process is cost effective, and allows to produce any shaped material.

Another advantage of our technique would be tuning the microstructure and mechanical properties of these composites by adjusting the degree of carbonation, sintering temperature and beginning particle size and pore size of CaSiO<sub>3</sub> compacts which provide capability to produce customized implant to get the best fit with host bone depending bone type, gender and age.

Our study proved that carbonation process effectively produced  $CaSiO_3-CaCO_3-SiO_2$  composites with high strength, toughness, as well as tunable density strength properties

and being commercially more applicable make carbonation process an ideal method in production of CaSiO<sub>3</sub> based bone implants.

#### **2.5.** Conclusion

Applying carbonation process to conventionally sintered CaSiO<sub>3</sub> definitely improved densification, compression and flexural strength and fracture toughness over only sintered CaSiO<sub>3</sub> ceramics. An increase in the relative density from 68.5 to 84.3 g/cm<sup>3</sup>, indicate that the compacts were densified after carbonation. The compression strength of 279 MPa and bending strength of 65.5 MPa, fracture toughness of 1.87 MPa.m<sup>1/2</sup> were achieved with the H1150LTS samples (sintered at 1150°C and then carbonated). The dense CaSiO<sub>3</sub>-CaCO<sub>3</sub>-SiO<sub>2</sub> ceramic composites produced by the carbonation of green (LTS) and sintered CaSiO<sub>3</sub> (H1100LTS and H1150LTS) having compressive strength and toughness properties similar to those of human cortical bone might be potential candidate as bioactive implant materials.

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of (c) HTS1150, (d) H1150LTS samples

Bulk density (ρ <sub>b</sub> , g/cm <sup>3</sup> )	Apparent density (ρa, g/cm <sup>3</sup> )	Tensile Strength (στ, MPa)	Compressive Strength (σc, MPa)	Young's Modulus (E, GPa)	Bending Strength (σc, MPa)	Fracture Toughness Mpa√m	Reference
1.85	2.0	60-160	130-180	3-30	-	2-12	[5]
-	-	90-190	130-290	-	-	-	[6]
-	-	-	100-230	7-30	50-150	2-12	[7]

Table 1 Properties of human cortical bone

Material	Forming Method	Heat treatment	Phases	Relative density (%)	Bending Strength (MPa)	Compressive Strength (MPa)	Elastic modulus (GPa)	Fracture Toughness (MPa m <sup>1/2</sup> )
Xonotlite [16]	(UP)50Mpa + (CIP) 200MPa	T <sub>sintering</sub> :1100°C t <sub>holding</sub> =24h T <sub>Annealing</sub> :1100C t <sub>holding</sub> =24h	W	65 68	42 51	-	-	-
	(UP) 14Mpa	900°C t <sub>holding</sub> =3h		65	3PB 5.4			
Synthetic W precipitation [11]	+ (CIP) 200MPa (15min)	$1100^{\circ}C$ $t_{holding} = 3h$ $t_{holding} = 5h$ $1200^{\circ}C$		81.4 89.5	65.9 95	-	-	-
	(101111)	t <sub>holding</sub> =3h		82.4	53.5			
Mineral W						26		
Mineral W milled	(UP) 500MPa	600°C/min 1400°C	PsW	-	-	48	-	-
Synthetic W Solid state [17]		t <sub>holding</sub> =2h				42		
Synthetic W chemical precipitation [18]	-	SPS 150-200°C/min 750°C, 40MPa t <sub>holding</sub> =5min	Amorph -W	89.35	3-PB 190	-	-	SENB 1.67
	-	150-200°C/min 950°C, 40MPa		75.6	100	-	-	1.52
Synthetic W chemical precipitation [19]	-	SPS 150-200°C/min 950°C, 40MPa t <sub>holding</sub> =5min	w	95	3-PB 290		-	SENB 2.0
	-	SPS 150-200°C/min 970°C, 40MPa t <sub>holding</sub> =5min	PsW	99	3-PB 65	-	-	SENB 0.5
W W,%20wt HA [1]	-	selective laser sintering	-	82.96 92.26	-	18.19 27.28	0.12 0.16	Vickers 1.19 1.43
Synthetic W (precipitation) W, W %50wt β-TCP [21]	(UP)10Mpa + (CIP) 200MPa	HTS 2°C/min 1100°C t <sub>holding</sub> =5h	W ½ W, ½ β-TCP	75.7 81.3	3PB 35.1 61.7	-	-	Not mentioned 0.6 0.9
Synthetic W (sol-gel) CaSiO <sub>3</sub> CaSiO <sub>3</sub> .5 mol% ZnO CaSiO <sub>3</sub> .5 mol% ZrO <sub>2</sub> [22]	(UP) 200MPa	5°C/min 950°C t <sub>holding</sub> = 0.5 h	-	-	28 36 42	-	8 11 17.5	-

Table 2 Properties of studied CaSiO<sub>3</sub> ceramics

UP: Uniaxial Press, CIS: Cold Isostatic Press, HTS: Conventional High Temperature Sintering,

SPS: Spark Plasma Sintering, SENB: Single-Edge Notch Beam

Sample	HTS	LTS
	T sintering (°C)	T carbonation (°C), P (psi)
HTS1100	1100	-
HTS1150	1150	-
HTS1200	1200	-
LTS	-	90, 20
H1100LTS	1100	90, 20
H1150LTS	1150	90, 20
H1200LTS	1200	90, 20

Table 3 Terminology used to define processed  $CaSiO_3$  ceramics

Sample	Wollastonite	Pseudowollastonite	Calcite	Aragonite	Vaterite	
Sampre	CaSiO3	CaSiO3	CaCO3	CaCO3	CaCO3	SiO2
Raw Material	97	0	1.1	0	0	1.6
HTS1100	97	0	0	0	0	3
HTS1150	90	10	0	0	0	0
HTS1200	3	97	0	0	0	0
LTS	40	0	22.5	28	4.5	5
H1100LTS	48	0	15	0	32	5
H1150LTS	43	9	14	0	31	3
H1200LTS	0	86	4	0	10	0

Table 4 Crystalline phase compositions of raw CaSiO<sub>3</sub>, and HTS, LTS and HLTS samples (wt %)

	λτga	λw	λc
LTS	52.98	52.68	52.59
H1100LTS	45.55	46.95	44.38
H1150LTS	36.36	37.3	36.69
H1200LTS	13.51	13.77	14.93

Table 5 Degree of carbonation for LTS and HLTS samples

Temperature (°C)	Pore size (µm)					
	HTS	LTS / HLTS				
RT		0.025 - 0.034				
1100	0.598	0.094 - 0.057				
1150	1.279	0.187 - 0.246				
1200	2.718	1.655 - 1.023				

Table 6 Pore size distribution of HTS, LTS and HLTS samples

	Degree of Carbonation	Relative Density	Open Porosity	Compressive Strength	Elastic Modulus	Bending Strength	Vickers Hardness	Fracture Toughness
	(λτga) mole %	%	%	MPa	GPa	MPa	GPa	Mpa√m
HTS1100	0	66.01±0.53	34.20	42.66±3.67	2.29	13.40±1.33	0.30±0.01	-
HTS1150	0	67.73±0.19	32.22	129.97±5.8	14.26	47.15±2.64	0.60±0.02	0.96±0.05
HTS1200	0	68.59±0.22	30.71	113.9±8.25	17.38	41.31±0.51	0.79±0.04	-
LTS	52.68±0.42	84.85±0.21	14.59	276.68±38.7	20.69	70.02±2.59	2.24±0.07	1.76±0.19
H1100LTS	45.00±0.78	84.25±0.64	15.35	253.4±12.76	18.88	61.01±3.51	1.98±0.03	1.61±0.15
H1150LTS	37.13±1.08	83.62±0.76	15.78	279.24±31.05	18.83	65.52±2.1	$1.71 \pm 0.07$	1.87±0.13
H1200LTS	12.56±1.34	71.92±0.57	27.33	120.08±26.7	18.68	34.06±3.08	0.75±0.01	-

Table 7 Summary of the properties of HTS, LTS, and HLTS processed  $CaSiO_3$  ceramics



Figure 1 X-ray diffraction spectra of the raw CaSiO<sub>3</sub>, HTS, LTS and HLTS samples.

Unmarked peaks; wollastonite, c; calcite, a; aragonite, v; vaterite, p; PsW



Figure 2 (a) Thermal analysis of HTS, LTS and HLTS samples



Figure 2(b) Degree of carbonation for HTS, LTS and HLTS samples



Figure 3 Relative Density of HTS, LTS and HLTS samples



Figure 4 (a) Pore size distribution of HTS, LTS and HLTS samples



Figure 4 (b) Porosity of HTS, LTS and HLTS samples



Figure 5 BET surface area of HTS, LTS and HLTS samples.

(Raw is raw CaSiO<sub>3</sub> while others are processed compacts)



Figure 6 (a) BSE image of Ion Beam Milled (IBM) surfaces of

(a) HTS1100, (b) HTS1150, (c) HTS1200 samples



Figure 6 (b) BSE image of Ion Beam Milled (IBM) surfaces of

(a-1) LTS, (b-1) H1100LTS, (c-1) H1150LTS, and (d-1) H1200LTS, and

EDS chemical mapping of IBM surfaces of

(a-2) LTS, (b-2) H1100LTS, (c-2) H1150LTS, and (d-2) H1200LTS

(Carbon; green, Oxygen; turquoise, Silicon; magenta and Calcium; red)



Figure 7 (a) Stress vs strain plot of HTS, LTS and HLTS samples



Figure 7 (b) Compressive strength of HTS, LTS and HLTS samples



Fig 7 (c) Compressive strength change by relative density of processed CaSiO<sub>3</sub>



Figure 8 Modulus in compression mode for HTS, LTS and HLTS samples



Figure 9 Bending strength of HTS, LTS and HTLS samples



Figure 10 Vickers hardness of HTS, LTS and HLTS samples



Fig 11 (a) Fracture toughness specimen and 3-point fixture configuration

(dimensions in mm), and fracture surface



Fig 11 (b) Load-time plot verifying slow crack growth behavior of the material

system



Figure 11 (c) Fracture toughness of HTS, LTS and HLTS samples



Figure 11 (d) Fracture surfaces of (a) HTS1150, (b) H1150LTS. Crack propagation

path of (c) HTS1150, (d) H1150LTS samples

## **Chapter 3: The Modulation of Soluble Factors via Carbonation**

## of CaSiO<sub>3</sub> Ceramics

Berra Beyoglu <sup>a</sup>, Yong Mao <sup>b</sup>, Stephanie Fung <sup>b</sup>, Paul Kim <sup>a</sup>, Richard E. Riman <sup>a</sup>,

<sup>a</sup> Rutgers University, Department of Materials Science and Engineering, 607 Taylor Rd. Piscataway NJ, 08854,USA

<sup>b</sup> New Jersey Center for Biomaterials, Rutgers, The State University of New Jersey, 145 Bevier Road, Piscataway, NJ 08854, USA

KEYWORDS Low temperature solidification (LTS), carbonation, hydrothermal reaction, high temperature sintering (HTS), wollastonite, bioactive ceramic, bioactive composite, ionic dissolution, solubility, osteoconductivity, osteoinductivity

#### Abstract

In this study, CaSiO<sub>3</sub> scaffolds have been processed by High Temperature Sintering (HTS) or Low Temperature Solidification (LTS, carbonation) or combinations thereof. The dissolution behavior, biocompatibility and osteoinductive potential of processed CaSiO<sub>3</sub> scaffolds were evaluated by Simulated Body Fluid (SBF) immersion, in-vitro cell proliferation and osteogenic differentiation tests, respectively. The ionic concentration of Ca and Si ions (soluble factors) in the SBF, after immersion of processed CaSiO<sub>3</sub> samples, indicated that carbonated green and sintered CaSiO<sub>3</sub> exhibited a decrease in the concentration of soluble factors, in contrast to the high ion dissolution from conventionally sintered CaSiO<sub>3</sub> This beneficial effect was achieved by the carbonation process repressing the solubility of the ions, thus maintaining them below toxic levels. Thus, carbonation process can be used to optimize soluble ion concentrations released from the CaSiO<sub>3</sub>. Scaffolds with significantly (p < 0.05) higher proliferation of mouse osteoblast progenitor cells (MC3T3) and osteogenic differentiation of human Mesenchymal Stem Cells (hMSC) were observed in scaffolds produced by carbonation of sintered CaSiO<sub>3</sub> at  $1150^{\circ}$ C, compared to conventionally sintered CaSiO<sub>3</sub> and osteoinductive 45S5 bioglass controls.

Our results suggest that CaSiO<sub>3</sub> ceramics processed by combination of HTS and LTS appear to be a potential osteoinductive bone implant material.

#### **3.1 Introduction**

The overall objective of this study is to produce a bioactive material with the potential to repair bone defects or bone injury sites. Bioactive materials as a class includes Ca-Si based glasses, glass-ceramics and (a range of other materials generally considered) ceramics. A common characteristic of the calcium-silicate bioactive materials is their release of soluble species, namely Si and Ca ions, henceforth referred as "soluble factors". Soluble factors can form a biologically active carbonated apatite layer on the implant surface, thus assisting in the creation of strong bone-implant interfaces. Above all, soluble factors can enhance cell proliferation [1], osteogenic differentiation [2][3][4][5] and promote new bone formation in-vivo [6][7].

Soluble factors, both organic and inorganic, can affect all stages of bone generation [8]. Bone morphogenetic proteins (BMP) are well known organic soluble factors. Depending on the type, BMP may possess the osteoinductive activity or only osteoconductivity[9]. Similarly, Ca and Si are inorganic soluble factors released from calcium-silicate based materials including bioglasses and glass-ceramics and ceramics. Depending on concentration of Ca and Si released from the implant material, it may possess osteoinductivity or only osteoconductivity[2]. Osteoconductive materials provide a scaffold upon which new bone grows. In case of bone injury, if a hard tissue gap is created, new bone growth is supported only along the bone-implant interface. Osteoconductive activity is observed on bone implant materials with high biocompatibility [10] including HA. calcium-phosphate (TCP), and calcium-silicate ceramics [11][12][13].

Osteoconductive calcium-silicate glasses and ceramics form apatite-like layer on the implant surface as a result of Ca and Si ion releases from the material. This apatite-like layer provides strong bonding at bond-implant interface. Osteoinduction is a basic biological mechanism that occurs regularly in the course of bone healing after a fracture. In presence of a correct stimulus, an undifferentiated mesenchymal cell (hMSC) transforms into a preosteoblast and provides regeneration of the bone tissue. This important process for bone healing is also referred to as bone induction [10]. Unlike osteoconductivity, an external material, such as an implant surface, is not a prerequisite for bone induction. However, studies revealed that some materials accelerate new bone formation by chemically stimulating undifferentiated (mesenchymal) cells located near the implant to differentiate into bone progenitor cells. Therefore, these materials were defined as "osteoinductive material" [2][14]. Introducing osteoinductive implants provides faster bone healing at the site of injury [11][15][16] by stimulating the body's own repair mechanisms to regenerate bone. Osteoinductive materials, in addition to their bonding to bone, different than osteoconductive materials, they have also ability to form bonds with soft tissues (ectopic sites), such as muscles [17]. Moreover, the use of osteoinductive implants is crucial in case of large bone defects, since the natural migration of osteoprogenitor cells does not suffice for fracture healing at the center of the implants [15]. Our goal in this study to produce an osteoinductive material to repair bone defects resulting from injury and disease and expedite the healing process.

45S5 bioglass is a well-studied bioactive glass composition. It is a unique due to its osteoinductivity [18][19][20][21][22]. 45S5 bioglass with Si ion concentration of <19 ppm in the medium stimulates (activates) osteogenic differentiation (osteoinductivity) [23][24].

Bioglasses can also be not osteoinductive if the release of soluble factors is not in the right range, due to a lack of ionic stimuli or extensively high ionic concentrations. Moreover, bioglass has poor mechanical properties, namely high brittleness, low strength and low fracture toughness. Thus, it cannot be used for load bearing applications. This drawback limits its use to very few clinical applications [17].

As a result, a bone implant material having both osteoinductivity and good biomechanical properties compatible with human cortical bone has not been discovered. In recent years, one of the promising materials for bone replacement is calcium-silicate ( $CaSiO_3$ ) due to its higher mechanical strength as compared to bioactive glasses. However, in comparison to 455S bioglass, CaSiO<sub>3</sub> ceramics having higher solubility produce higher concentrations of soluble factors in the relevant test media [25][4]. Especially pseudowollastonite (PsW) [16] has higher dissolution rate of soluble factors as a result of PsW having an unstable "threering silicate" crystal structure in comparison to the stable "chain-silicate" structure of wollastonite. The released Ca ions combine with  $PO_4^{3-}$  ions present in the blood or in-vitro test medium and heterogeneously nucleate and grow as hydroxyapatite (HA,  $Ca_{10}(PO_4)_6(OH)_2)$  on CaSiO<sub>3</sub>. Higher dissolution leads to faster bone-like apatite formation. This bone-like apatite layer facilitates implant-bone bonding (osteoconductivity) of bioactive material [26]. However, high Si concentrations, >120 ppm, released from a variety of silicate biomaterials were reported as cytotoxic to osteoblasts causing programmed cell death (apoptosis) [27][4]. The concentration of soluble factors released from calcium-silicate based material is a critical parameter defining if material is osteoinductive or only osteoconductive, or toxic to body. CaSiO<sub>3</sub> ceramics release high ion concentrations, whereas osteoinductivity of the material requires much

lower concentrations of soluble factors. In order to activate osteoinductivity of CaSiO<sub>3</sub>, the concentration of soluble factors released from sintered (HTS) CaSiO<sub>3</sub> should be decreased. In this study, we will investigate if we can produce CaSiO<sub>3</sub> ceramics having osteoinductivity comparable to 45S5 bioglass. The osteoinductivity of the CaSiO<sub>3</sub> scaffold can be created by controlling the concentrations of soluble factors released from the implant material.

In-vitro assessment of implant's biocompatibility and osteoinductive potential is done by observing cell proliferation of bone cells and osteogenic differentiation of immature mesenchymal stem cells (hMSCs) in contact with the implant [14]. Sarmento et al. reported in-vitro cell studies on PsW and demonstrated an increase in both the rate and total numbers of bone nodules formed. However, no improvement was detected in either cell proliferation or differentiation [28]. Ni et al. reported that  $CaSiO_3$  is superior to Tricalcium Phosphate (TCP) in cell attachment, proliferation, and differentiation [5]. Zhang et al. compared in-vitro activity of hMSCs on fine versus coarse grain PsW. They reported fine-grained PsW showed less cell attachment and lower viability than coarsegrained PsW surface. This was attributed to the higher Si ion dissolution (>120 ppm) from fine grained PsW. As mentioned earlier, these concentrations are cytotoxic. They reported osteogenic differentiation occurred on both surfaces, but the lack of an osteoinductive control group prevents this work from being conclusive [25]. Zhang et al. studied cell growth and osteogenic differentiation of hMSCs on two CaSiO<sub>3</sub> polymorphs, PsW (β-CaSiO<sub>3</sub>) and wollastonite ( $\alpha$ -CaSiO<sub>3</sub>). They reported that the PsW polymorph which initially reached to cytotoxic Si levels is more osteoinductive than the wollastonite polymorph. [4]. Lin reported strontium-substituted CaSiO<sub>3</sub> scaffolds, in comparison to

pure CaSiO<sub>3</sub> scaffolds, have better osteoinductivity to enhance early bone formation and stimulate osteogenic differentiation of bone marrow mesenchymal stem cells derived from ovariectomized rats (rBMSCs-OVX) [29]. However, none of the work stated above has studied osteoinductivity of CaSiO<sub>3</sub> based ceramics and 45S5 bioglass in terms of osteoblast cell proliferation and differentiation.

In this study, we will investigate if high solubility of CaSiO<sub>3</sub> can be altered by phase chemistry to better control its solubility. This can be accomplished by partial conversion of highly soluble CaSiO<sub>3</sub> into less soluble CaCO<sub>3</sub> and a silica-rich amorphous phase. Here, we propose the carbonation process can improve biocompatibility and osteoinductivity properties of HTS CaSiO<sub>3</sub> ceramics by limiting high amount of Ca and Si ion release from CaSiO<sub>3</sub> as well as the resulting pH change.

This carbonation process is defined as Low Temperature Solidification (LTS carbonation) method. LTS is a new densification technique invented by Riman et al. and it has been reported that LTS process improved strength of calcium-silicate based ceramics [30][31]. In our earlier study, we proved that LTS via carbonation process densifies HTS CaSiO<sub>3</sub> and significantly enhances the mechanical properties of CaSiO<sub>3</sub> ceramics cost effectively as summarized in Table 1 [32]. Schematic of LTS process is shown in Fig. 1. Li et al. represented LTS process in 3 steps:

"(a) a porous compact having a network of interconnected pores is formed using conventional ceramic processing methods,

(b) the porous compact is then infiltrated with a fluid composed of reactive cations and/or anions,
(c) a hydrothermal reaction causes the part of the porous compact to dissolve and react with the fluid to form a product that reactively grows while filling the pore space."[31]

The LTS method has produced core-shell structures including cores of partially carbonated acicular CaSiO<sub>3</sub> grains surrounded by SiO<sub>2</sub>-rich layers that are in turn encapsulated by CaCO<sub>3</sub> particles [30][31][32]. Reaction products of LTS process composed of CaCO<sub>3</sub> and SiO<sub>2</sub> rich amorphous phase are chemically more stable and biologically acceptable. Moreover, we expect CaCO<sub>3</sub> and SiO<sub>2</sub> rich amorphous phase reaction products encapsulating highly soluble CaSiO<sub>3</sub> to address the drawback of high concentration of soluble factors released from the CaSiO<sub>3</sub>. LTS method is promising in control of CaSiO<sub>3</sub> solubility since highly soluble CaSiO<sub>3</sub> is partially consumed and surrounded by less soluble reaction products during carbonation reaction. Thus, carbonation process can be used to optimize soluble ion concentrations released from CaSiO<sub>3</sub>.

The objective of this study is to experimentally investigate if the extraction of Ca and Si ions released from calcium silicate biomaterials can be controlled with concomitant carbonation of the material to produce particulate composite structures has potential to be osteoinductive. Such materials will be valuable in bone repair and in the expedition of bone healing processes.

## **3.2 Experimental Methods**

## **Experimental Strategy**

The strategic direction of this work is to perform experiments that yield an understanding of the effect of High Temperature Sintering (HTS) and Low Temperature Solidification

(LTS) processes on dissolution behavior, in-vitro cell proliferation, and osteogenic differentiation of CaSiO<sub>3</sub> materials. First, CaSiO<sub>3</sub> compacts were processed by HTS method. Afterwards, the LTS carbonation process were used to densify  $CaSiO_3$  green bodies and further densify HTS CaSiO<sub>3</sub>. HTS CaSiO<sub>3</sub> were used as control samples. Hereafter, the term HLTS were employed to designate CaSiO<sub>3</sub> ceramics prepared by "high temperature sintering followed by low temperature solidification". Prefixes of 1100, 1150, and 1200 for HTS and HLTS terms represent sintering temperatures applied during sintering (Table 2). Crystalline phase analysis, the degree of carbonation were monitored to characterize samples before the evaluation of their dissolution and in-vitro behavior. In order to evaluate dissolution behavior of these ceramics, CaSiO<sub>3</sub> compacts processed by HTS, LTS, and HLTS were immersed into Simulated Body Fluid (SBF) for different time periods. After SBF soaking, the concentration of Ca, Si and P ions, pH change of the SBF and weight loss of samples were monitored. Alternatively, the equilibrium solubility of CaSiO<sub>3</sub> and reaction products in SBF was predicted using OLI Analyzer modeling software. Thin-Film X-Ray Diffraction (TF-XRD) and Field Emission Scanning Electron Microscopy (FESEM) methods were used to probe for apatite formation resulting from SBF soaking. In-vitro cell studies were performed to evaluate the composite cytotoxicity, cell adhesion, and proliferation in the presence of mouse osteoblast progenitor cells (MC3T3) and its osteoinductive ability via osteogenic differentiation of human mesenchymal stem cells (hMSCs). After preliminary evaluation of CaSiO<sub>3</sub> samples via cell proliferation test, HTS1100 and H1100LTS samples were eliminated and control 45S5 bioglass was included to following osteogenic differentiation test.

#### **Raw Materials**

Mineral based wollastonite (NYAD<sup>®</sup> 400, NYCO Minerals Inc., Willsboro, NY) was used as the CaSiO<sub>3</sub> source. It has an average particle size of ~9 μm as determined by a laser particle size analyzer (Zetasizer, Nano ZS, Malvern Instruments Ltd., Malvern, Worcestershire, UK) (see Appendix A). Industrial grade CO<sub>2</sub> (AirGas Inc., Piscataway, NJ) was used for the carbonation process. Water was filtered through a Progard<sup>®</sup> 2 and Qguard<sup>®</sup> 1 purification system (EMD Millipore, MA) prior to use.

#### **Powder Processing**

#### *CaSiO*<sub>3</sub> *compacts*—*Preparation and High temperature sintering (HTS)*

Powder compacts of ~13 mm of diameter and ~13 mm of length were prepared by dry pressing using Automatic Carver Press (Model 4532, Wabash, IN) using a pressure of 150 MPa. The compacts were dried overnight in a drying oven at 90°C.

Powder compacts were sintered in a box furnace (CM Furnaces Inc., Rapid Temp Furnace, Bloomfield, NJ) at 1100, 1150, and 1200°C respectively in air, for 2 h, with heating rate of 2°C/min and cooling rate of 10°C/min.

## Low Temperature Solidification (LTS) on Green and Sintered CaSiO<sub>3</sub> Compacts

The green and sintered ceramic compacts were reacted at 90°C with 20 psig CO<sub>2</sub> input in a pressure steamer (All American #75x, Wisconsin Aluminum Foundry Co., Manitowoc, WI) for a minimum of 19 h. After the reaction, the samples were dried at 90°C for 24 h. During the LTS process, carbonation reaction formed shown in Eqn. 1:

$$CaSiO_{3(s)} + CO_{2(g)} = CaCO_{3(s)} + SiO_{2(s)}$$
 (1)

#### **Characterization Methods**

#### **Characterization of prepared samples**

X-ray diffraction (XRD) analysis was performed using a Bruker D8 Discover (Bruker AXS Inc., Madison, WI) with  $Cu_{K\alpha}$  radiation ( $\lambda = 1.514$  Å), parallel beam in the range 10-80° (2 $\theta$ ) with a 0.018° step size and 0.5-s dwell time. PDF numbers of 97-020-1537 (wollastonite 1A-CaSiO<sub>3</sub>), 97-008-7716 (pseudowollastonite (high temperature phase of CaSiO<sub>3</sub>)), 97-028-0991 (aragonite-CaCO<sub>3</sub>), 97-001-6710 (calcite-CaCO<sub>3</sub>), 97-001-5879 (vaterite-CaCO<sub>3</sub>), and 98-0039830 (SiO<sub>2</sub>) published by the International Centre for Diffraction Data (ICDD) was used to identify the phases. Thermal Gravimetric Analysis (TGA) was performed on TA Q500 (TA Instruments, New Castle, DE) by heating from room temperature to 1000°C at 10°C/min under nitrogen with a flow rate of 100ml/min. Weight loss in the range of 200-800°C [33] corresponds to the amount of CO<sub>2</sub> released as a result of carbonate decomposition in accordance with Eqn. 2:

$$CaCO_{3(s)} = CaO_{(s)} + CO_{2(g)} \uparrow$$
<sup>(2)</sup>

The weight percentage of CO<sub>2</sub> ( $m_{CO_2}$ %) is proportional to the degree of carbonation ( $\lambda_{TGA}$  (%)) as shown in Eqn. 3:

$$\lambda_{\text{TGA}} (mol \%) = (m_{\text{CO}_2}\%) \times \left(\frac{M_{CaSiO_3}}{M_{CO_2}}\right)$$
(3)

$$\lambda_{\text{TGA}} (mol \%) = \left(1 - \left(\frac{(m_i - m_{CO2})}{(m_i)}\right)\right) \times \left(\frac{M_{CaSiO_3}}{M_{CO_2}}\right)$$

 $m_i = initial \ sample \ mass \ in \ grams$  $m_{CO_2} = mass \ of \ CO_2 \ in \ grams \ released \ from \ sample$ 

$$M_{CaSiO_3} = molecular weight of CaSiO_3, 116.16 g/mole$$
  
 $M_{CO_2} = molecular weight of CO_2, 44.01g/mole$ 

Specimens 3 mm thick discs were cut from the cylindrical samples using a precision saw (Pace Technologies, Tucson, AZ) for both the SBF ion dissolution studies and the in-vitro cell tests. These discs were ultrasonically washed in deionized water, and subsequently dried in air for 12 h at 90°C.

#### Ion dissolution and apatite formation on CaSiO<sub>3</sub> ceramics in SBF

Dissolution behavior and apatite forming ability of CaSiO<sub>3</sub> ceramics processed by HTS, LTS, and HLTS were assessed by infiltrating CaSiO<sub>3</sub> ceramics in Simulated Body Fluid (SBF). SBF was prepared according to the method reported by Kokubo (Table 3) [34] to possess similar ion concentrations to those found in human blood plasma [34]. The preparation was conducted by dissolving reagent grade NaCl, NaHCO<sub>3</sub>, KCl, K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, MgCl<sub>2</sub>·6H<sub>2</sub>O, CaCl<sub>2</sub>, and Na<sub>2</sub>SO<sub>4</sub> in deionized water, and buffered at pH = 7.4 with tris(hydroxymethyl) aminomethane (CH<sub>2</sub>OH)<sub>3</sub>CNH<sub>2</sub> and 1 M HCl at 36.5°C. After ultrasonic washing in deionized water, the discs were sterilized in an autoclave for 20 min. Each sample was then soaked in 50 ml of SBF with a pH comparable to that of human blood plasma and maintained at 36.5°C for 1, 7, 14, and 21 days. Osteoinductive 45S5 Bioglass ® [45% SiO<sub>2</sub>, 24.5% CaO, 24.5% Na<sub>2</sub>O and 6% P<sub>2</sub>O<sub>5</sub> (wt. %)], (MO-SCI Corporation, Rolla, MO) discs were used as a control for comparison.

After soaking in SBF, the samples were rinsed with deionized water, and dried at room temperature. The apatite formation on the specimen surfaces was examined by Thin-Film X-Ray Diffraction (TF-XRD), Field Emission Scanning Electron Microscopy (FE-SEM) and Energy Dispersive Spectroscopy (EDS). Specimens were examined both before and

after immersion in SBF using TF-XRD (ADVANCE D8, Bruker-AXS, Madison, WI, USA) at a glancing beam angle of 1° with Cu-Kα X-ray radiation from a source operating at 40 kV and 40 mA. The surface morphologies were observed by FESEM using a Zeiss Sigma FESEM (Carl Zeiss, Oberkochen, Germany) at an accelerating voltage of 15 kV and working distance of 8.5 mm. Sample surfaces were sputter coated with carbon (Electron Microscopy Sciences, model# EMS 150T ES, Hatfield, PA). The distribution of elements was analyzed using an EDS detector on the FESEM.

Dissolution behavior of CaSiO<sub>3</sub> scaffolds processed by HTS, LTS, and HLTS was evaluated by measuring changes in the ion concentrations of Ca and Si in the SBF solution after immersion using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES; Perkin Elmer, Optima 7300DV, Shelton, CT). The changes in the pH were determined using an electrolyte-type pH meter (HI 2221, Hanna Instruments, Woonsocket, RI). The dissolution of the scaffold material in SBF was followed by concomitant apatite formation as well as weight loss of the scaffolds. Weight loss of samples as a function of time of dissolution in the SBF were calculated from their measured, overnight oven-dried at 90°C, initial weight and final weight after SBF immersion. Sample weights were determined for specimens dried at 90°C overnight. The weight loss, ICP, and pH values reported are averages of three-fold replication.

#### Cytotoxicity assay

To evaluate if possible toxic substances leached from the CaSiO<sub>3</sub> samples, cytotoxicity tests were performed that used mouse osteoblast progenitor cells (MC3T3) exposed to the eluents of CaSiO<sub>3</sub> scaffolds processed by HTS, LTS and HLTS. CaSiO<sub>3</sub> discs were sterilized by autoclaving for 20 min. To prepare the eluents, each disc was placed in a well

of a 24-well tissue culture plate (Denville Scientific, Deville, NJ) and 1 ml of alpha-MEM complete medium (alpha-MEM medium+10% fetal bovine serum) (Gibco, Grand Island, NY) was added to each disc. After incubation at 37°C for 24 h, the medium (eluent) from each disc was collected and transferred to a corresponding well of new 24-well plate. Mouse osteoblast progenitor cells MC3T3 (P2-P4) (ATCC, Manassas, VA) cultured in 10 cm tissue culture dish (Denville Scientific, Deville, NJ) were trypsinized by adding 1 ml of 0.25% trypsin-EDTA (Gibco, Grand Island, NY) and incubated for 5 min. Cells were resuspended in alpha-MEM complete medium at  $5 \times 10^{5}$ /ml. The cells of 0.2 ml were added to the wells containing eluent or alpha-MEM complete medium (control) and the plates were incubated at 37°C with 5% CO<sub>2</sub> and >95% humidity for 24 h. Afterwards, the eluent was removed and each well was washed with Phosphate Buffered Saline (PBS) (Gibco, Grand Island, NY) twice. The cell viability was measured using alamarBlue assay, which measures the natural reducing power of living cells to convert resazurin to the fluorescent molecule, resorufin (BUF012B AbD Serotec). 300 µl of medium containing 10% alamar Blue was added to each well, after incubation for 1 h at 37°C, 100 µl of medium from each well was transferred to a 96-well plate (Denville Scientific, Deville, NJ). The fluorescence intensity at 590 nm of resorufinat excited at 560nm was read using a TeCan fluorometer (Infinite M200, Männedorf, Switzerland). The relative viability of cells in each eluent was normalized to the viability of cells cultured in alpha-MEM complete medium.

#### **Cell Adhesion and Proliferation on discs**

MC3T3 cells adhesion to the composite discs was evaluated as follows: Sterilized discs were washed with Phosphate Buffered Saline (PBS) three times. Composite discs were placed in the wells of a 24-well plate and 1 ml of MC3T3 cells were seeded onto each disc

or Tissue Culture Polystyrene Surface (TCPS) at  $2.5 \times 10^4$ /well in alpha-MEM complete medium. After incubation for 24 h, the discs were transferred to a new wells and the cells were washed with PBS twice. 500 µl of medium containing 10% alamarBlue was added to each well and incubated at 37°C for 1 h. 100 µl of medium from each well was transferred to a 96-well plate and the fluorescence intensity at 590 nm of resorufinat excited at 560 nm was measured using a TeCan fluorometer (M200). The cell adhesion on each disc at 24 h was calculated as the relative viability of cells on disc normalized to the viability of cells on TCPS.

To monitor the cell proliferation, after the alamarBlue assay, cells on composite discs or TCP were washed with PBS twice and recovered in complete medium for 2 h. Once the medium was removed, 1 ml of fresh medium was added to the cells. Cell proliferation was evaluated after incubation for 1, 7, 14 and 20 days by measurement of the cell viability using alamarBlue assay. The relative viability of cells on discs or TCPS at each time point was normalized to its own viability at day 1.

#### Cell differentiation on discs

Osteoinductivity of a material is measured by the material's ability to induce osteogenic differentiation, or differentiate stem cells to osteoblasts *in vitro* [14]. The ability of HTS, LTS, and HLTS processed CaSiO<sub>3</sub> scaffolds to induce cell differentiation was evaluated. Samples of 12mm in diameter and 3 mm in thickness were prepared. Osteoinductive 45S5 Bioglass ® [45% SiO<sub>2</sub>, 24.5% CaO, 24.5% Na<sub>2</sub>O and 6% P<sub>2</sub>O<sub>5</sub> (wt %)], (MO-SCI Corporation, Rolla, MO) discs with 12 mm in diameter and 1 mm in thickness were used as a control for comparison of osteoinductivity. Sterilized discs were placed in the wells of a 24-well non-treated tissue culture plate. 1 mL of human mesenchymal stem cells (hMSCs,

Lonza, passage <6) were seeded onto each disc at 2.5 x 10<sup>4</sup> cells/well in complete alpha-MEM medium (alpha-MEM medium+10% fetal bovine serum). Samples were incubated for 24 h. After 24 h, the discs were transferred to a fresh plate. Cells were cultured for 14 days, with media changes every 2-3 days. On day 14, alkaline phosphatase (ALP) activity was quantified using an Alkaline Phosphatase Activity Fluorometric Assay Kit (Biovision, Milpitas, CA) according to manufacturer's instructions. Briefly, enzymatic activity in the cell lysates was measured by the cleavage of a phosphate group of a non-fluorescent substrate, 4-methylumbelliferyl phosphate disodium salt (MUP), by ALP, which results in a fluorescent signal. The intensity of the fluorescent signal can be used to measure ALP activity using a standard curve. ALP activity was normalized to dsDNA content, which was measured using the Quant-iT Picogreen dsDNA Assay Kit (ThermoFisher, Waltham, MA). This assay uses a fluorescent dye that specifically binds to double stranded DNA.

#### **Statistical analysis**

Data is represented as the mean  $\pm$  standard error of the mean. Statistical analysis was performed in Prism 7.0 (Graphpad Software, La Jolla, CA) using one-way ANOVA with post-hoc Tukey's test. *p*<0.05 was considered statistically significant.

## **3.3 Results**

#### Phase Analysis

XRD patterns of green, HTS, LTS and HLTS processed CaSiO<sub>3</sub> are shown in Fig 2. Before carbonation, wollastonite (low temperature phase of CaSiO<sub>3</sub>) was observed for green and sintered samples at 1100 (HTS1100) and 1150°C (HTS1150). Pseudowollastonite (PsW), a high temperature phase of CaSiO<sub>3</sub>, was the only primary phase detected for samples

sintered at 1200°C. The transformation of wollastonite to PsW is known to occur in the range of 1125±10°C [35], although the conversion temperature depends to some degree on the chemistry of the wollastonite, increasing with the presence of meta-silicate solid solutions formed by introduction of Mg or Fe ions into the structure [36]. After carbonation, all emerging peaks corresponded to CaCO<sub>3</sub> phases of calcite, aragonite and vaterite. Property data related to the degree of carbonation of CaSiO<sub>3</sub> ceramics processed by HTS, LTS, or HLTS are given in Table 1[32]. The carbonated green CaSiO<sub>3</sub> (LTS) showed a degree of carbonation of 52 % whereas pre-sintering the wollastonite prior to carbonation (HLTS) reduced the degree of carbonation to 45-12 %, depending on the sintering temperatures in the range of 1100-1200°C. The degree of carbonation decreased with increasing sintering temperature of CaSiO<sub>3</sub> generated by the sintering process [32].

#### Ion dissolution and apatite formation of HTS, LTS, and HLTS CaSiO<sub>3</sub> scaffolds

The XRD patterns of CaSiO<sub>3</sub> ceramics processed by HTS, LTS, and HLTS before and after soaking in SBF for 21 days are shown in Fig. 3 (a-b). The characteristic peaks of carbonated apatite ( $2\theta = 25.7^{\circ}$  and  $2\theta = 31.7^{\circ}$ ) (97-016-7228) were detected on CaSiO<sub>3</sub> sintered at 1150 and 1200°C (HTS1150, HTS1200) and their corresponding carbonated forms (H1150LTS, H1200LTS), and 45S5 bioglass control. Sintered and carbonated (HLTS) materials showed relatively low intensity apatite peaks compared to only sintered forms of CaSiO<sub>3</sub> (HTS). Observation of apatite peaks accompanied with significant reduction in the diffraction intensity of the original phases of the ceramics indicated the apatite layer formation on the sample surface. The intensity of the apatite peaks increased with increasing sintering temperature of the HTS specimens. TF-XRD patterns of sample surfaces shown in Fig. 4

illustrate apatite nucleation as a function of SBF immersion period. The apatite formation on the sample surface appeared after (i) 1 day for 45S5 bioglass, (ii) 7 days for HTS1200 CaSiO<sub>3</sub>, and (iii) 14 days for HTS11500 CaSiO<sub>3</sub>. Thus apatite formation on sintered and then carbonated (HLTS) samples was delayed compared to their only sintered (HTS) forms. In addition, the lower intensity of apatite peaks indicates that apatite formation is limited in carbonated materials compared to only-sintered CaSiO<sub>3</sub>.

SEM micrographs and EDS-microanalysis of the sample surfaces after the immersion in SBF for 21days are shown in Fig. 5. Apatite formation was observed on HTS1150, HTS1200, H1150LTS, and H1200LTS CaSiO<sub>3</sub> and 45S5 bioglass surfaces, which confirmed XRD results discussed earlier. A continuous apatite layer with typical "cauliflower" morphology covering the entire surface of the sample was observed on HTS1150, HTS1200, H1200LTS, and the bioglass, whereas H1150LTS showed only incipient apatite formation that failed to entirely cover the sample surface. Apatite formation was not detected on (a) the reacted green  $CaSiO_3$  (LTS), (b) low temperature sintered CaSiO<sub>3</sub> (HTS1100) and (c) low temperature sintered then reacted CaSiO<sub>3</sub> (H1100LTS) during the test period. SEM/EDS microanalysis showed that the newly formed apatite layer contained mainly Ca, P and low levels of Si with the atomic ratio of calcium to phosphorus around 1.66 after 21 days of soaking (Appendix B). The EDS analysis of carbonated samples showed that CaSiO<sub>3</sub> preferentially dissolves in SBF leaving the carbonation products, the silica rich phase and calcite, remained as an interconnected porous structure (Fig. 7). Preferential dissolution of CaSiO<sub>3</sub> can be observed more clearly on H1200LTS samples due to faster dissolution of PsW compared to wollastonite phase in H1150LTS samples. The morphological changes of apatite forming surfaces after 1, 7, 14

and 21 days of immersion in SBF are shown in Fig. 6, 7 and 8. Confirming the XRD results, the apatite layer formation appeared after (i) 1 day for 45S5 bioglass, (ii) 7 days for HTS1200 CaSiO<sub>3</sub>, and (iii) 14 days for HTS11500 CaSiO<sub>3</sub>(Fig 8). Both XRD and SEM analysis revealed that apatite formation on sintered and carbonated (HLTS) CaSiO<sub>3</sub> occurred more slowly and in more limited amounts compared to the only sintered (HTS) CaSiO<sub>3</sub>.

Changes in soluble Ca and Si concentrations in the SBF solution after 21 days of immersion are shown in Fig. 9 and Table 4 as a function of sintering temperature. With increasing sintering temperature, the concentration of Ca and Si species released from the samples increased. By dissolution of HTS CaSiO<sub>3</sub>, Si concentrations increasing from 77 to 85 ppm and Ca concentrations increasing from 220 to 560 ppm, with increasing sintering temperature. The difference in dissolution behavior of wollastonite -slow and incongruentand PsW -faster and congruent- explained with the difference in their silicate anion structure [37]. CaSiO<sub>3</sub> composed of covalently bonded Si-O tetrahedra network formers and weakly bonded network-modifying Ca cations. While wollastonite composed of silicate chains, PsW composed of three membered silicate rings. In case of wollastonite, weakly bonded network-modifying Ca cations are released to solution relatively faster and hydrogen ions from the solution takes their place. Covalently bonded network formers remain behind to form a hydrogen-enriched leached layer that dissolves more slowly. In addition, the hydrated silica in the leached layer undergoes reconstruction reactions become more stable [37][38]. On the contrary, for PsW, once Ca ion leave the structure, three membered silicate rings can't be stable, release into the solution as monosilicic acid, resulted in higher ion concentrations released from PsW.

The changes in soluble Ca and Si concentrations in the SBF solution as a function of immersion time are shown in Fig. 10. The Ca and Si concentrations in SBF increased over the exposure time indicating partial dissolution of material. The literature reports that high Si concentrations released from PsW (100-120 ppm) [39], and Ca concentrations >400 ppm are cytotoxic to osteoblasts [40]. Ca and Si ionic concentrations released from carbonated samples decreased compared to their only sintered forms. The Ca ion concentrations released from sintered and then carbonated (HLTS) CaSiO<sub>3</sub> in comparison to only sintered (HTS) CaSiO<sub>3</sub> are (i) 147 ppm for H1100LTS vs 220 ppm for HTS1100 (ii) 347 ppm for H1150LTS in comparison to 501 ppm for HTS1150, and (iii) 406 ppm for H1200LTS in comparison to ~560 pm for HTS1200. The Si ion concentrations released from HLTS samples in comparison to HTS samples are (i) 43 ppm for H1100LTS in comparison to 77 ppm for HTS1100, (ii) 68 mm for H1150LTS in comparison to 83 ppm for HTS1150, (iii) 84 ppm for H1200LTS in comparison to 86 ppm for HTS1200. The Ca and Si ion concentrations of carbonated samples decreased proportional to their degree of carbonation. Equilibrium solubility of Ca and Si ions from CaSiO<sub>3</sub> and carbonation products, CaCO<sub>3</sub> and amorphous silica, in SBF calculated by the thermodynamic modelling are shown in Fig. 10. According to the modelling, the equilibrium solubility of  $CaCO_3$  and amorphous silica are lower than that of  $CaSiO_3$  although detailed analysis requires consideration of the common ion effects. Thus, for carbonated samples, the interacting dissolution effects of all three phases -CaSiO<sub>3</sub>, CaCO<sub>3</sub> and amorphous silicain SBF medium must be considered.

The pH values of SBF solutions after sample infiltration and weight loss of samples in SBF are shown in Fig. 11 as a function of immersion time. After 1 day immersion, the pH

increased from its original value of 7.4 to 8.1 for control 45S5 bioglass and to ~7.9 for all CaSiO<sub>3</sub> samples. General trend for CaSiO<sub>3</sub> samples is increasing pH by immersion time until 14 days then constant pH for the remaining time of the test. During the dissolution of silicates, pH increase is expected due to acid consumption (hydroxide formation) during cation/proton exchange [41]. The pH increase became more gradual at longer soaking times due to slower dissolution of  $CaSiO_3$  as pH increases [42]. The pH increased proportionately to the increase in Ca and Si ions released from the samples by increasing sintering temperatures. Sintered and then carbonated (HLTS) samples shows similar trend but the increase in the pH was more gradual (limited). The pH of 45S5 bioglass immersed SBF, after sharp increase to 8.1 at day 1, decreased to pH 8 and remained almost constant during the rest of the monitoring period of 21 days. The changes of pH in the SBF solution followed a trend similar to the ion release profile. The pH increased with increasing ion release. According to the pH data, the apatite formation was observed once the samples reached to pH 8. Hence, LTS, HTS1100, and H1100LTS samples, having the pH values lower than pH 8 showed no apatite formation. The pH of these samples showed a negligible increase or almost constant during the rest of the monitoring period of 21 days. Similar to pH, weight loss of processed CaSiO<sub>3</sub> samples and control bioglass increased proportionately to the increase in Ca and Si ions released from the samples which increased with immersion time and increasing sintering temperature.

#### In-Vitro Cell Studies on HTS, LTS, and HLTS CaSiO<sub>3</sub> scaffolds

At 24 h of culture, cell (MC3T3) incubation with material extracts HTS, LTS, and HLTS processed CaSiO<sub>3</sub> scaffolds showed no cytotoxicity effect of CaSiO<sub>3</sub> (Fig. 12).

Cells showed significantly higher (p < 0.05) adhesion to LTS CaSiO<sub>3</sub> and H100LTS CaSiO<sub>3</sub> samples compared to other CaSiO<sub>3</sub> samples and similar to TC plates within the first 24h of culture (Fig. 13). According to the literature, cell adhesion is sensitive to the morphology. In addition, the chemistry of the substrate is important [5] where Ca concentrations <240 ppm promote cell adhesion [40][25]. Therefore, a large amount of dissolution from the surface and the local pH increase could prevent cell adhesion in the course of early immersion periods. Better cell adhesion can be observed on the samples having relatively low degree of dissolution. However, very low ion concentrations are not enough to stimulate osteoblast proliferation and osteogenic differentiation of cells.

Proliferation of MC3T3 on CaSiO<sub>3</sub> discs processed by HTS, LTS, and HLTS as well as on tissue culture plastic is shown in Fig 14. The changes in the number of viable cells on the scaffolds after 1, 7, 14 and 20 days in the culture were quantitatively assessed. MC3T3 cultured on H1150LTS discs exhibited higher cell proliferation at each time point of the culture compared to those on the other samples and tissue culture plastic. Statistical analysis of 20 day proliferation data showed proliferation of H1150LTS discs is significantly higher (p< 0.05) compared to other CaSiO<sub>3</sub> samples. Viability on H1150LTS discs increased from 1 to 4, 10 and 11 fold, over day 1, steadily during the culture period. In-vitro osteogenic differentiation was assessed in terms of the alkaline phosphatase (ALP) activities of the hMSCs at 14 days. Osteoblastic differentiation of hMSCs on CaSiO<sub>3</sub> disc surfaces processed by HTS, LTS and HLTS in growth medium is shown in Fig. 15. ALP activity on H1150LTS discs was significantly greater than that on other samples and control 45S5 bioglass. Briefly, for HTS CaSiO<sub>3</sub>, the concentrations of soluble factors in SBF increased markedly with increasing sintering temperature. The adhesion and proliferation of osteoblastic cells and osteogenic differentiation of hMSCs decreased with increasing concentrations of soluble factors. For LTS and HLTS CaSiO<sub>3</sub>, the concentrations of soluble factors were found to decrease in proportion to the degree of carbonation (Fig 16). Significant increase in proliferation of osteoblastic cells and osteogenic differentiation of hMSCs on H1150LTS samples indicated that the amount of the soluble factors released from H1150LTS samples, Si ion concentration of ~49 ppm and Ca ion concentration of ~237 ppm at 14 day SBF soaking, should be in an ideal range to activate stem cells for their differentiation to osteoblast cells during the culturing.

## 3.4 Discussion

The experimental results of this study support our proposed hypothesis that the carbonation process improves biocompatibility and osteoinductivity properties of HTS CaSiO<sub>3</sub> ceramics by limiting high Ca and Si ion concentrations released from CaSiO<sub>3</sub> to reach optimum ion concentrations. When the carbonation process was used for green CaSiO<sub>3</sub> and sintered CaSiO<sub>3</sub> samples, soluble factor concentrations were found to decrease with increasing degree of carbonation. The partial consumption of highly soluble CaSiO<sub>3</sub> by carbonation leads to the formation of less soluble CaCO<sub>3</sub> and amorphous silica accounts. These reaction products play an important role in decreasing the concentration of soluble factors.

Cell proliferation and osteoinduction requires an optimum concentration of soluble factors be present in order to encourage bone growth. Whereas high concentration of soluble factors are toxic to cells, too low concentrations of Ca and Si ions net no impact on cell proliferation and osteoinductivity. Therefore, in order to have osteoinductive material the concentration of soluble factors released from sintered (HTS) CaSiO<sub>3</sub> should be decreased to reach optimum levels. Based on our experimental results that proved the higher degree carbonation leads to the lower ion concentration released from the CaSiO<sub>3</sub> composite, carbonation process can be used to optimize soluble ion concentrations released from the CaSiO<sub>3</sub>. Of the carbonated samples, only one range of soluble factors reached by H1150LTS samples provided superior cell proliferation and osteogenic differentiation compared to all other samples.

According to thermodynamic modeling, the equilibrium solubility of Ca and Si ions from CaSiO<sub>3</sub> is much greater than solubility limit of Ca ions from CaCO<sub>3</sub> and Si ions from amorphous silica (Fig 10). However, since CaSiO<sub>3</sub> is not fully consumed and converted to less the soluble CaCO<sub>3</sub> and amorphous silica, thermodynamically there should be no decrease in the solubility of the composite. Thus, if enough time is given, it is expected carbonated samples should reach similar Ca and Si ion equilibrium solubility levels with only sintered CaSiO<sub>3</sub>. Therefore, it can be concluded that carbonation process have not altered thermodynamic solubility of the material.

Thermodynamic solubility of CaSiO<sub>3</sub> and carbonation products, CaCO<sub>3</sub> and amorphous silica from literature, in water at 25°C, is given in Table 5 [41][43][44][45][46][47]. However, due to lack of literature based on experimental equilibrium solubility limits of CaSiO<sub>3</sub> and CaCO<sub>3</sub> and amorphous silica phases in SBF salt solution at pH of 7.4 and temperature of 37°C, thermodynamic solubility of the system may not be evaluated accurately. Here, equilibrium solubility of Ca and Si ions from CaSiO<sub>3</sub>, CaCO<sub>3</sub>, amorphous silica and HA were predicted using the thermodynamic modeling (Fig 10) to evaluate the

system thermodynamically. According to the modeling, Ca ionic concentrations released from sintered CaSiO<sub>3</sub> (HTS1150 and HTS1200) samples reached to the equilibrium solubility limit of CaSiO<sub>3</sub>. However, Ca ionic concentrations released from carbonated samples have not reached the equilibrium solubility level of CaSiO<sub>3</sub> during the test period. Therefore, it can be concluded that the Ca dissolution from carbonated samples should be kinetically limited. The same trend was expected to be seen for Si ions released from sintered and carbonated samples. However, the modeling gave very low Si solubility levels from  $CaSiO_3$  and amorphous silica, which are not acceptable because it conflicts with our experimental data as well as literature [41][44][47]. In addition, Si equilibrium solubility from CaSiO<sub>3</sub>, according to the model, is below Si ion concentration released from the less soluble bioglass. However, 45S5 bioglass is known by its low Si ion solubility in comparison to highly soluble  $CaSiO_3$ . This mismatch might be due to either equilibrium solubility limits reached by the modeling may not representative of real conditions, or other factors such as presence of impurities or extra silica, dissolved CO<sub>2</sub> in the solution or interacting dissolution of multiple phases affected the dissolution.

HA formation following the ion dissolution in SBF can be thermodynamically evaluated based on the equilibrium solubility limits based on the model. The concentration of Ca ions released from processed CaSiO<sub>3</sub> are above the equilibrium solubility limit of calcite and HA. Therefore, since the saturation limit is exceeded for CaCO<sub>3</sub> and HA, these phases can thermodynamically form. Formation of HA is more favorable, due to a lower standard energy of formation for HA,  $\Delta H_f^o$ =-3,212 kcal/mole [43], compared to that of CaCO<sub>3</sub>,  $\Delta H_f^o$ =-289 kcal/mole [41]. HA formation was observed on the surfaces of HTS1150 and HTS1200 samples, while no HA was observed on HTS1100. HA formation on HTS1100 samples might be kinetically limited due to lower concentrations of soluble ions from HTS1100. Therefore, if enough immersion time given, HA formation is expected to be seen on this sample. Following the carbonation process, even though there is a reduction in the ion concentrations, the Ca ion concentrations released from CaSiO<sub>3</sub> and CaCO<sub>3</sub> exceeded the equilibrium solubility of HA. Hence, HA formation on surfaces of the carbonated samples is expected, however, it may proceed slower due to lower ion concentrations compared to only sintered CaSiO<sub>3</sub>

Decrease in Ca and Si ion concentrations following carbonation process might be due to dissolution of ions from the composite are kinetically limited. Assuming the dissolution of soluble ions from carbonated samples are kinetically limited, kinetic factors that may be effective on reaching lower ion concentrations of soluble factors following carbonation process were discussed here. Relative quantity of phases, microstructure, surface area and pH might be parameters affect dissolution kinetics and resulted in beneficial concentrations of soluble factors in H1150LTS, but which were not effective in the other materials.

*The relative quantities* of highly soluble CaSiO<sub>3</sub> and lower solubility reaction products, CaCO<sub>3</sub> and amorphous silica, can affect dissolution kinetics. The relative quantities of reaction products and CaSiO<sub>3</sub> matrix were quite different depending on the level of sintering which in turn affected the degree of subsequent carbonation. Depending on relative quantities of highly soluble CaSiO<sub>3</sub> or less soluble CaCO<sub>3</sub> and amorphous silica in the composite, the ionic concentrations of soluble factors in the SBF variates. Considering the degree of carbonation is not dependent on phase of CaSiO<sub>3</sub>, (wollastonite and PsW show no difference in reactivity during carbonation), monotonically decrease in degree of carbonation with sintering temperature, apparently due to coarsening of the morphology and 85 % reduction in the surface area. For H1200LTS samples, decreasing degree of carbonation provides more calcium silicate to enable greater levels of soluble factors appear in the solution. In contrast, H1100LTS samples sintered at lowest temperature have very high degree of carbonation, therefore the lower CaSiO<sub>3</sub> amount in those samples resulted in a very low concentration of soluble factors compared other carbonated samples. However, H1150LTS having moderate level of CaSiO<sub>3</sub> resulted in medium concentration of soluble factors.

*Microstructure* appears to be an important parameter affecting dissolution kinetics of the carbonated samples. The reaction products due to their surround of CaSiO<sub>3</sub> matrix may act as a barrier slowing down the dissolution of CaSiO<sub>3</sub>. For the lowest temperature sintered material prior to carbonation (H1100LTS), the microstructure is comprised of an array of discrete fine CaSiO<sub>3</sub> crystals that are highly reactive during the carbonation step and thus generate a protective carbonate/amorphous silica reaction layer around discrete particles. Soluble factor release in SBF is thus highly reduced. On the other hand, the highest temperature sintered then carbonated material (H1200LTS) exhibits a much coarser interconnected network-like structure of  $CaSiO_3$  that is less reactive in the carbonation environment. Yet, this less carbonated material still has enough CaSiO<sub>3</sub> to release high quantities of soluble factors. Within the scope of this study the intermediate sintered then carbonated material (H1150LTS) optimizes the solubility. The fine CaSiO<sub>3</sub> particles sinter to form a network-like structure that is sufficiently fine-structured to produce moderate reactivity during carbonation, leading to a structure that partially protects the CaSiO<sub>3</sub> from the SBF to yield a biologically favorable level of soluble factors.

*Surface area* might be another parameter affecting the dissolution kinetics of carbonated samples. Samples with higher surface area should yield higher concentration of soluble factors. Before carbonation, the surface area of CaSiO<sub>3</sub>, decreased by 85 % with increasing sintering temperature. On the contrary, the concentration of soluble ions increased with increasing sintering temperature due to faster dissolution of ions from PsW. Therefore, it can be concluded microstructural change of CaSiO<sub>3</sub> affects dissolution kinetics of CaSiO<sub>3</sub> much strongly in comparison to surface area change. In case of carbonation, final surface area of carbonated samples increased compared to surface area of highly soluble CaSiO<sub>3</sub> compacts prior to carbonation. Increase in surface area is proportional to the degree of carbonation due to formation of reaction products, CaCO<sub>3</sub> and silica rich leached layer [32][48][37]. Due to increase in the surface area arisen from formation of less soluble phases, there is no observable contribution to the concentration of soluble factors (dissolution kinetics).

*Interacting dissolution* appeared to be a parameter affecting ion concentrations of carbonated samples. Studies on silicate minerals reported mineral-fluid interface are complex and still not fully understood [49]. In our study, involvement of CaCO<sub>3</sub> and silica rich amorphous phases in addition to CaSiO<sub>3</sub>, and the usage of SBF salt solution compared to pure water brings much more complexity. Even though the ion concentration data is not enough to explain the interacting dissolution effects of all three phases in SBF medium, here, we discussed possible dissolution behavior based on individual dissolution characteristics of each phase depending on pH.

Dissolution kinetic also changes based on pH of the solution. For sintered samples, CaSiO<sub>3</sub> was the only compound available. Wollastonite is an alkaline earth silicate mineral that

dissolves faster in acidic solutions than neutral ones due to abundance of H ions in the solution. CaSiO<sub>3</sub> dissolution increased the pH arising from acid consumption (hydroxide formation) [42]. Partial dissolution of CaSiO<sub>3</sub> in SBF is shown in Eq. 4

$$CaSiO_{3(s)} + 3H_2O_{(l)} \leftrightarrow Ca^{2+}{}_{(aq)} + 2OH^- + H_4SiO_{4(aq)}$$
 (4)

Monosilicic acid, H<sub>4</sub>SiO<sub>4</sub>, is the predominant form of silica in solutions until pH of 9. The literature reports that CaSiO<sub>3</sub> dissolves incongruently due to preferential release of Ca ions from CaSiO<sub>3</sub> surface and concomitant formation of silica-rich leached layers at pH values lower than 8.5. Si–OH groups on the surface form by the exchange of Ca ions by hydrogen ions (protons) from the solution [49] [50]

By carbonation, CaSiO<sub>3</sub> was partially consumed and replaced by less soluble reaction products, CaCO<sub>3</sub> and amorphous silica. Due to decrease in the CaSiO<sub>3</sub> amount available in samples resulted in a decrease in the concentration of soluble factors released from CaSiO<sub>3</sub> and gradual increase of the pH. CaCO<sub>3</sub> being alkaline mineral similar to CaSiO<sub>3</sub> dissolves slower in weakly alkaline pH of SBF due to lack of H<sup>+</sup>. CaCO<sub>3</sub> dissolution consumes H<sup>+</sup> ion leading to increase in the pH. However, since CaCO<sub>3</sub> is less soluble than CaSiO<sub>3</sub>, limited increase in the pH is expected. Carbonate dissolution reaction is shown below [41];

$$CaCO_{3(s)} + H_2O_{(l)} \leftrightarrow Ca^{2+}{}_{(aq)} + OH^- + HCO_3^-$$
 (5)

Amorphous silica is acidic oxide and has limited solubility in the pH range of 2-9 [41][51]. Similar to dissolution process of silicates, dissolution of silica is based on hydrolysis of Si-0-Si bonds. The anionic silicate surfaces protonate and dissociate to release monosilicic acid (H4SiO4) [37].

$$\operatorname{SiO}_{2\,(\mathrm{s})} + 2\operatorname{H}_{2}\operatorname{O}_{(\mathrm{l})} \leftrightarrow \operatorname{H}_{4}\operatorname{SiO}_{4\,(\mathrm{aq})} \tag{6}$$

Solubility of amorphous silica is reported as pH independent in acidic and weak alkali solutions until pH of 9. Then solubility of silica increases dramatically at pHs over 9 [41][44][51]. However, pH of SBF solution does not reach high solubility generating limit. Considering pH of the SBF solution is in the range of 7.4-8.5, dissolution rate of CaSiO<sub>3</sub>, CaCO<sub>3</sub> and amorphous silica are expected to be limited at that pH range.

Based on the behavior of each individual phase, a possible mechanism involving the interaction of CaSiO<sub>3</sub>, CaCO<sub>3</sub>, and amorphous silica phases with SBF solution might be;

(i) Ca<sup>2+</sup> ions are released from CaSiO<sub>3</sub> and limited release from CaCO<sub>3</sub> increases pH,

(ii) Hydrolysis of surface of carbonation product amorphous silica and Si–OH surfaces of CaSiO<sub>3</sub> and release of H<sub>4</sub>SiO<sub>4</sub> to solution (Eqn.6),

(iii) Partial condensation of ≡Si-OH groups in the surface occurs in layers depleted in Ca ions,

$$\equiv \text{Si} - \text{OH} + \text{HO} - \text{Si} \equiv \rightarrow \equiv \text{Si} - \text{O} - \text{Si} \equiv + \text{H}_2\text{O}$$
(7)

(iv) Once released the  $Ca^{2+}$  and  $OH^{-}$  ions exceed the solubility of the apatite in SBF, apatite nucleation and crystallization on favorable silica rich surfaces occurs as reported in the literature [52].

$$10Ca_{(aq)}^{2+} + 6HPO_{4(aq)}^{2-} + +80H_{(aq)}^{-} \leftrightarrow Ca_{10}(PO_4)_6(OH)_{2(s)} + 6H_2O$$
(8)

*Surface chemistry* might be a parameter affecting dissolution kinetics of carbonated samples. Surface charges of sintered and carbonated  $CaSiO_3$  in SBF test conditions were predicted based on point zero of charge (PZC) –the pH at which solid surface charges are zero– of each phases. Under acidic conditions, pH values below the pH<sub>PZC</sub>, mineral surface

becomes more positive and the solution becomes more alkaline. Under alkaline conditions, pH values above this  $pH_{PZC}$ , mineral surface becomes less positive and the solution becomes more acidic. For sintered samples, CaSiO<sub>3</sub> was the only material that existed. Point zero of charge (PZC) of wollastonite reported as ~PH 7 [53]. So, the surface of CaSiO<sub>3</sub> in pH in the range of 7.4-8.5 might be negatively charged but close to zero charge. For carbonated samples, in addition to CaSiO<sub>3</sub>, surface chemistry of calcite and amorphous silica should be considered. Point zero of charge (PZC) of calcite reported as ~PH 8-9 [54]. Thus, the surface of  $CaCO_3$  might be positively charged but close to the zero charge. Point zero of charge (PZC) of amorphous silica reported as ~PH 3.5 [53]. Thus, the surface of amorphous silica might be negatively charged. For carbonated samples, net surface charge of the composite might be negative due to high negative charging on amorphous silica and negligible amount of charging of other phases. As a result, SBF solution tend to be more acidic. According to our data, the pH of carbonated samples, in comparison to only sintered  $CaSiO_3$ , was relatively lower. It is believed that the decrease in the amount of dissolved CaSiO<sub>3</sub> was the main reason for limited increase in the pH. In addition, surface charges contribute to reach lower pH values. However, it should be considered the effect of surface charges should be limited due to low surface area of the material (maximum  $\sim 2m^2/g$ ), thus surface charges should not induce a major change in the solution pH.

Considering these possible kinetic factors, it can be interpreted that carbonation process kinetically limits the dissolution of Ca and Si ions from the CaSiO<sub>3</sub> based composite.

The H1150LTS samples having great biocompatibility and osteoinductivity properties can expedite the healing process to repair these bone defects. In addition to biochemical compatibility and osteoinductivity requirement, biomechanical compatibility of implant

with natural bone is substantial parameter that bone implant should possess. Our previous study demonstrated that carbonation of (a) green  $CaSiO_3$  (LTS), (b)  $CaSiO_3$  sintered at 1100 (H1100LTS) and (c) CaSiO<sub>3</sub> sintered at 11500 (H11500LTS) similarly enhanced densification and mechanical strength and toughness compared to the only sintered (HTS) CaSiO<sub>3</sub>. Due to relatively lower degree of carbonation of sintered then carbonated (HLTS)  $CaSiO_3$  samples compared to those of only carbonated green (LTS)  $CaSiO_3$ , it may have been expected that HLTS samples to have lower densification and less enhancement in mechanical properties compared to LTS CaSiO<sub>3</sub>. However, high temperature sintering process (especially at sintering temperature of 1150°C) and formation of lower theoretical density phase of CaCO<sub>3</sub> (vaterite takes the place of aragonite) contributed to reach similar densification and mechanical properties with HLTS CaSiO<sub>3</sub> scaffolds. By applying carbonation process, compressive strength reached to 279 MPa, flexural strength increased to 65.5 MPa and toughness rose to  $1.87 \text{ MPa.m}^{1/2}$  which is close to that of cortical bone and about two times of the conventionally sintered CaSiO<sub>3</sub> recorded in our study and also reported previously [1]. Out of LTS, H1100LTS and H1150LTS samples, all providing similarly promising mechanical properties, only H1150LTS optimizes both biomechanical and biochemical compatibility and osteoinductivity. This study demonstrates that H1150LTS samples produced by carbonation of sintered CaSiO<sub>3</sub> at 1150°C meet the essential conditions by compromising right concentration of released soluble factors reaching greater cell proliferation and osteogenic differentiation as well as having promoted mechanical properties. H1150LTS CaSiO<sub>3</sub> having improved mechanical properties, biocompatibility, and superior osteoinductivity, may be a potential candidate to be used as bioactive bone implant.

In-vitro cell tests provided primary evaluation to explore osteoinductive potential of the material. However, more comprehensive in-vitro cell test and in-vivo ectopic bone formation of material should be investigated to approve this material is classified as osteoinductive.

## 3.5 Conclusion

Our in-vitro cell results revealed the concentrations of soluble factors significantly influenced osteoinductivity. Utilizing of the carbonation process to sintered CaSiO<sub>3</sub> addresses the drawback of high ion dissolution from sintered CaSiO<sub>3</sub> by reducing the concentration of soluble factors from toxic levels. Significantly greater cell proliferation of MC3T3 on H1150LTS compared to other CaSiO<sub>3</sub> substrates and significantly greater osteogenic differentiation of hMSCs on H1150LTS compared to other CaSiO<sub>3</sub> substrates and osteoinductive 45S5 bioglass control indicated soluble factors of H1150LTS scaffolds may be in an optimal range of ion concentrations for enhancing biocompatibility and osteoinductivity. In-vitro cell proliferation and osteogenic differentiation tests can point out promising concentrations of soluble factors, Si ion concentration of ~49 ppm and Ca ion concentration of ~237 ppm at 14 day SBF soaking, released from H1150LTS CaSiO<sub>3</sub> that can stimulate cell activation for bone generation.

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of CaCO <sub>3</sub> )

	Degree of Carbonation (λτGA) mole %	Relative Density %	Open Porosity %	Compressive Strength MPa	Elastic Modulus GPa	Bending Strength MPa	Vickers Hardness GPa	Fracture Toughness Mpa√m
HTS1100	0	66.01±0.53	34.20	42.66±3.67	2.29	13.40±1.33	0.30±0.01	-
HTS1150	0	67.73±0.19	32.22	129.97±5.8	14.26	47.15±2.64	0.60±0.02	0.96±0.05
HTS1200	0	68.59±0.22	30.71	113.9±8.25	17.38	41.31±0.51	0.79±0.04	-
LTS	52.68±0.42	84.85±0.21	14.59	276.68±38.7	20.69	70.02±2.59	2.24±0.07	1.76±0.19
H1100LTS	45.00±0.78	84.25±0.64	15.35	253.4±12.76	18.88	61.01±3.51	1.98±0.03	1.61±0.15
H1150LTS	37.13±1.08	83.62±0.76	15.78	279.24±31.05	18.83	65.52±2.1	1.71±0.07	1.87±0.13
H1200LTS	12.56±1.34	71.92±0.57	27.33	120.08±26.7	18.68	34.06±3.08	0.75±0.01	-

Table 1 Summary of the properties of CaSiO<sub>3</sub> ceramics processed by HTS, LTS, or HLTS methods

Sample	HTS	LTS	
	T sintering (°C)	T carbonation (°C), P (psi)	
HTS1100	1100	-	
HTS1150	1150	-	
HTS1200	1200	-	
LTS	-	90, 20	
H1100LTS	1100	90, 20	
H1150LTS	1150	90, 20	
H1200LTS	1200	90, 20	

Table 2 Terminology used to define processed  $CaSiO_3$  ceramics

	Concentration	Concentration $(10^{-3} \text{ mole})$ in				
Ion	SBF	Blood plasma				
	(pH 7,40)	(pH 7,2 to 7,4)				
Na <sup>+</sup>	142,0	142,0				
$K^+$	5,0	5,0				
Mg <sup>2+</sup>	1,5	1,5				
Ca <sup>2+</sup>	2,5	2,5				
Cl	147,8	103,0				
HCO <sub>3</sub> <sup>-</sup>	4,2	27,0				
HPO4 <sup>2-</sup>	1,0	1,0				
SO4 <sup>2-</sup>	0,5	0,5				

Table 3 Ion concentrations of SBF and human blood plasma
	Ca	Si
HTS1100	220.62	77.09
HTS1150	501.02	82.77
HTS1200	558.92	85.6
LTS	126.5	39.75
H1100LTS	147.12	43.03
H1150LTS	347.72	68
H1200LTS	406.52	84.1
45S5 Bioglass	126.32	30.04

Table 4 Ca and Si ion concentrations (ppm) released from HTS, LTS and HLTS samples after 21 day SBF soaking as a function sintering temperature

	Solubility			
Mineral	in H <sub>2</sub> O at 25°C (mol/l)	in SBF at 37°C (g l <sup>-1</sup> ) HSC software		
CaSiO3 (Wollastonite)		1.9 10 <sup>-5</sup> [45]		
CaSiO3 (PsW)		3.4 10 <sup>-5</sup> [45]		
CaCO <sub>3</sub> (Calcite)	3.36 10 <sup>-9</sup> [46] 4.5x10 <sup>-9</sup> [41]			
CaCO <sub>3</sub> (Aragonite)	$6x10^{-9}[41]$			
SiO2 Amorphous Silica	120ppm [41] [47] 100ppm [44]			
$Ca_{10}(PO_4)_6(OH)_2$	3.7 x10 <sup>-58</sup> [43]			
(Hydroxyapatite)				

Table 5 Solubility of related phases reported in the literature



Figure 1 Schematic of g-rHLPD process

"(A) Dried porous CaSiO<sub>3</sub> preform; (B) Partially wet CaSiO<sub>3</sub> preform; (C) Final densified monolithic solid. Steps 1 to 4 represent the carbonation-densification process occurring in an individual pore: Step (1) Partially wet pore with CO<sub>2</sub>; Step (2) Diffusion, dissolution and dissociation of CO<sub>2</sub>; Step (3) Dissolution of CaSiO<sub>3</sub> by hydrogen ions; Step (4) Precipitation of solids. After the completion of step 4, the process takes place continuously following steps 2-4 until various kinetic factors slow down the process (e.g., thick SiO<sub>2</sub> reaction layers)"[30]



Figure 2 X-ray diffraction spectra of the raw, HTS, LTS and HLTS processed CaSiO<sub>3</sub>



Figure 3 TF-XRD patterns of HTS (a), LTS and HLTS processed CaSiO<sub>3</sub> and control 45S5 bioglass discs (b) before (gray) and after (black) 21 day SBF infiltration



Figure 4 TF-XRD patterns of sample surfaces showed apatite nucleation after SBF infiltration for time period of 1 day (light grey), 7 days (grey), 14 days (dark grey), and 21 days (black)



Figure 5 SEM images of HTS, LTS or HLTS processed  $CaSiO_3$  scaffolds after soaking in SBF for 21 days (Some NaCl precipitated from the SBF were detected in the EDS spectra)



Figure 6 SEM images of the surfaces of CaSiO<sub>3</sub> scaffolds sintered at 1150°C (HTS1150) and 1200°C (HTS1200) after soaking in SBF for 1, 7, 14 and 21 days

(Some NaCl precipitated from the SBF were detected in the EDS spectra)



Figure 7 SEM images of the surfaces of  $CaSiO_3$  scaffolds carbonated after sintering at 1150°C (H1150LTS) and 1200°C (H1200LTS) after soaking in SBF for 1, 7, 14 and 21 days. (Some NaCl precipitated from the SBF were detected in the EDS spectra)



Figure 8 SEM images of the surfaces of 45S5 bioglass scaffolds after soaking in SBF for 1, 7, 14 and 21 days



Figure 9 Ca and Si concentration of SBF after 21 day sample infiltration as a function sintering temperature



Figure 10(a) Ca, (b) Si ionic concentration change by infiltration time, and equilibrium solubility limits of Ca and Si from CaSiO<sub>3</sub> and CaCO<sub>3</sub> and amorphous silica calculated by a thermodynamical modelling



Figure 11(a) The pH change in the SBF, after HTS, LTS, or HLTS processed CaSiO<sub>3</sub> discs immersed, (b) The weight loss of HTS, LTS, or HLTS processed CaSiO<sub>3</sub> discs immersed in SBF solution



Figure 12 Cytotoxicity results for HTS, LTS, and HLTS processed CaSiO<sub>3</sub> discs and tissue culture polystyrene ((TCP) control)



Figure 13 Adhesion of osteoblastic cells to HTS, LTS, and HLTS processed CaSiO<sub>3</sub> discs and tissue culture polystyrene (TCP). \* Significantly higher (p< 0.05) cell adhesion to LTS CaSiO<sub>3</sub> and H100LTS CaSiO<sub>3</sub> ceramics compared to other CaSiO<sub>3</sub> samples and similar/close to TC plates within the first (24h) of culture.



Figure 14 Cell Proliferation of MC3T3 on HTS, LTS, and HLTS processed CaSiO<sub>3</sub> discs and tissue culture polystyrene (TCP) for 1, 7, 14, 20 days.\*Significant difference (p< 0.05) compared to other CaSiO<sub>3</sub> samples for the Day 20 proliferation data.



Figure 15 Cell differentiation of hMSCs on HTS, LTS and HLTS processed  $CaSiO_3$  discs. \*Significant difference (p< 0.05) compared to other  $CaSiO_3$  samples and control 45S5bioglass



Figure 16 Ca and Si ion Concentration change depending degree of carbonation (mole % of CaCO<sub>3</sub>)

### **Chapter 4: Conclusions**

CaSiO<sub>3</sub>-CaCO<sub>3</sub>-SiO<sub>2</sub> ceramic composites produced by processing CaSiO<sub>3</sub> ceramics via HTS and LTS methods successfully reached the mechanical and biological properties essential for bioactive bone implantation, thus fulfilling our goal in this study.

Applying LTS process to conventionally sintered CaSiO<sub>3</sub> definitely improved densification, compression and flexural strength and fracture toughness over only sintered  $CaSiO_3$  ceramics. The degree of carbonation of sintered then carbonated (HLTS)  $CaSiO_3$ samples decreased with increasing sintering temperature of HTS CaSiO<sub>3</sub> due to decrease in their surface area. However, high temperature sintering process (especially at sintering temperature of 1150°C) and formation of lower theoretical density phase of CaCO3 (vaterite takes the place of aragonite) contributed to reach densification and mechanical properties similar to those of LTS  $CaSiO_3$  scaffolds. Carbonated (a) green  $CaSiO_3$  (LTS), (b) CaSiO<sub>3</sub> sintered at 1100 (H1100LTS) and (c) CaSiO<sub>3</sub> sintered at 11500 (H11500LTS) samples possessed similarly enhanced densification and mechanical strength and toughness compared to the only sintered (HTS) CaSiO<sub>3</sub>. By applying carbonation process, compression strength of 279 MPa and bending strength of 65.5 MPa, fracture toughness of 1.87 MPa.m<sup>1/2</sup> were achieved. The dense CaSiO<sub>3</sub>-CaCO<sub>3</sub>-SiO<sub>2</sub> composites produced by the carbonation of green or sintered CaSiO<sub>3</sub> at 1100 and 1150°C possessed compressive strength and toughness properties similar to those of human cortical bone.

Applying LTS process to sintered CaSiO<sub>3</sub> addressed the drawback of high ion dissolution from sintered CaSiO<sub>3</sub> by lowering the concentration of released Ca and Si ions from toxic levels. Soluble factor concentrations found to decrease with increasing degree of carbonation. Significantly greater cell proliferation of MC3T3 on H1150LTS compared to other CaSiO<sub>3</sub> substrates and significantly greater osteogenic differentiation of hMSCs on H1150LTS compared to other CaSiO<sub>3</sub> substrates and osteoinductive 45S5 bioglass control indicated soluble factors of H1150LTS scaffolds may be in an optimal range of ion concentrations, for enhancing cell proliferation and osteogenic differentiation.

Out of LTS, H1100LTS and H1150LTS samples, all providing similarly promising mechanical properties, only H1150LTS compromises both biomechanical and biochemical compatibility and osteoinductivity. This study demonstrates that H1150LTS samples produced by carbonation of sintered CaSiO<sub>3</sub> at 1150°C meet the essential conditions by compromising right concentration of released soluble factors reaching greater cell proliferation and osteogenic differentiation as well as having promoted mechanical properties. H1150LTS CaSiO<sub>3</sub> having improved mechanical properties, biocompatibility, and superior osteoinductivity, may be a potential candidate to be used as bioactive bone implant.

### **Chapter 5: Future Work**

One promising application of our technique would be tuning the microstructure and mechanical properties of these composites by adjusting the degree of carbonation, sintering temperature and beginning particle size and pore size of CaSiO<sub>3</sub> compacts which provide capability to produce customized implant to get the best fit with host bone depending bone type, gender and age. Exploratory research on tuning material properties by changing the parameters above should be conducted.

In-vitro cell studies provided preliminary evaluation of if the material is promising for bone replacement applications. The in-vitro cell results indicated better biocompatibility and the potential osteoinductivity of H1150LTS CaSiO<sub>3</sub>. Next step research should focus on more comprehensive in-vitro studies to investigate long term effects. Ultimately, in-vivo studies should be performed to validate our findings in the complex body conditions. In addition, in-vivo bone formation in ectopic sites should be observed to approve this material is classified as osteoinductive.

# Appendix

## Appendix A

CHEMICAL COMPOSITION: CaSiO3				
COMPONENT	TYPICAL VALUE (%)			
CaO	46.36			
SiO <sub>2</sub>	51.60			
Fe <sub>2</sub> O3	0.77			
Al <sub>2</sub> O3	0.40			
MnO	0.15			
MgO	0.15			
TiO <sub>2</sub>	0.05			
K <sub>2</sub> O	0.02			
Wt. Loss (1000°C)	0.50			

Figure A 1 Chemical composition of raw  $CaSiO_3$ 



Figure A 2 Particle size distribution of raw CaSiO<sub>3</sub>



Figure A 3 Computed phase diagram of CaSiO<sub>3</sub>. Five polymorphs of the CaSiO<sub>3</sub> metasilicate (wollastonite, pseudowollastonite, walstromite, wollastonite-II, Caperovskite) (Belmonte, D., Ottonello, G., Zuccolini, M. V., Santos, R. L., Horta, R. B., Pereira, J., & Colaço, R. (2017). Ab initio-assisted assessment of the CaO-SiO 2 system under pressure. Calphad, 59, 12.)

#### **Appendix B**

EDS spectrum of HTS, LTS, HLTS  $CaSiO_3$  and 45S5 bioglass surfaces after 21 days of soaking is shown as weight percent of elements. The newly formed apatite layer contains mainly Ca, P. Samples showing apatite  $Ca_{10}$  (PO<sub>4</sub>)<sub>6</sub> (OH)<sub>2</sub> formation supposed to have:

- Ca/P mole ratio =  $(10/6) \approx 1.67$
- Ca/P weight ratio =  $(10 \text{ mole}*40.078 \text{ gr/mole}) / (6 \text{ mole}*30.97 \text{ g/mole}) \approx 2.16$

	Ca	Р	Si	Ca/P
HTS1100	29.09	0.01	20	NA
HTS1150	29.92	13.20	0.47	2.26
HTS1200	37.59	14.82	0.85	2.53
LTS	19.43	0.26	4.75	NA
H1100LTS	23.22	0.10	0.68	NA
H1150LTS	28.71	12.98	0.36	2.21
H1200LTS	23.56	10.66	0.20	2.21
45S5 Bioglass	25.92	12.24	0.16	2.12

Table B. Weight % of Ca, P, Si elements collected via EDS and weight ratio of Ca to P



Figure B-1 EDS spectrum of HTS sample surfaces







Figure B-3 EDS spectrum of LTS sample and 45S5 bioglass surfaces