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Biologic Potential of Calcium Phosphate Biopowders Produced via Decomposition Combustion Synthesis

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Abstract

The aim of this research was to evaluate the biologic potential of calcium phosphate (CaP) biopowders produced with a novel reaction synthesis system. Decomposition combustion synthesis (DCS) is a modified combustion synthesis method capable of producing CaP powders for use in bone tissue engineering applications. During DCS, the stoichiometric ratio of reactant salt to fuel was adjusted to alter product chemistry and morphology. In vitro testing methods were utilized to determine the effects of controlling product composition on cytotoxicity, proliferation, biocompatibility and biomineralization. In vitro, human fetal osteoblasts (ATCC, CRL-11372) cultured with CaP powder displayed a flattened morphology, and uniformly encompassed the CaP particulates. Matrix vesicles containing calcium and phosphorous budded from the osteoblast cells. CaP powders produced via DCS are a source of biologically active, synthetic, bone graft substitute materials

Keywords

Decomposition combustion synthesis; Bone tissue engineering; Calcium phosphate; Bioactivity; Osteoblasts

1.0 Introduction

Combustion synthesis is an overarching term coined for self-sustaining exothermic reactions that utilize internally generated energy [1] to rapidly produce a desired product. Traditional combustion synthesis methods utilize solid-state reactions of reactant powders [2] whereas decomposition combustion synthesis (DCS) requires the aqueous decomposition and

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subsequent reconstitution of the reactants into a gelatinous foam prior to ignition [3, 4]. Calcium phosphate DCS is a self-propagating high-temperature synthesis (SHS) process that utilizes the heat generated by the exothermic reaction of urea, calcium nitrate and ammonium phosphate to generate and sustain the propagation of a combustion wave [5–7] through the reactants.

DCS is a combustion synthesis technique that has been used to produce many advanced materials, including calcium phosphate (CaP) [3, 8]. An advantage of CaP DCS reactions is that reactant salt and fuel stoichiometries can be modified to produce CaP powders with diverse chemistries and morphologies [9] allowing the final product to be customized to specific manufacturing requirements. Additionally, product synthesis time is significantly lower than traditional synthesis techniques such as wet chemistry precipitation [10–14] or solid-state reactions [15–17].

Synthetic CaP materials currently used in bone tissue engineering applications are comprised of hydroxyapatite (HA) [18, 19], tricalcium phosphate (TCP)[20] or mixtures with varying percentages of HA and TCP[21–25]. Biphasic, triphasic and multiphasic CaPs are commonly utilized in reconstruction of bone defects [25] and it has been demonstrated that the bioactivity of biphasic CaPs can be directly controlled via manipulation of the CaP composition [21, 23, 26]. In addition to calcium and phosphate, bone mineral can contain carbonate ions [12, 27–29] and trace elements [28, 29].

An advantage of CaP DCS reactions is that product composition can be controlled. Specifically, reactant stoichiometry can be controlled to produce products with specific bi-, tri- or multiphasic compositions. Additionally, the CaP DCS reaction system could be modified to include constituents that enhance the mechanical, chemical and biological properties of the synthesized biopowders e.g. AgNO_3 [30] or ionic Ag [31] for antimicrobial effects, Mg^{2+} and F^- [32] for increased microhardness and cellular response. Whereas post synthesis heat treatment [13, 14] could be applied to the final products to produce pure (single) phase CaP powders. Subsequent to interaction with the human body, a CaP implant with biomimetic properties and compositional similarity to natural bone may reduce healing time, improve tissue integration and improve long term *in vivo* function [33, 34].

DCS represents a synthesis technique for the production of biologically active CaP biopowders that can be used as components in medical devices, bone cements, coatings and bone tissue engineering applications. The intent of this research is to examine the effects of altering DCS reaction stoichiometry on the biologic potential of CaP biopowders synthesized via DCS and to quantify the effects of controlling reaction parameters (e.g. reactant stoichiometry) on biologic activity.

2.0 Materials and Methods

2.1 Decomposition Combustion Synthesis Procedure

A detailed description of the DCS procedure has been given elsewhere [8]. Briefly, salts of calcium nitrate tetrahydrate [$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$], ammonium phosphate [$(\text{NH}_4)_2\text{HPO}_4$] and urea [$\text{CO}(\text{NH}_2)_2$] were dissolved in 10ml of de-ionized water in a beaker and heated on a hot

plate for 20min. During heating, the hydrolyzed reactants were vaporized until a white foam formed. Then the beaker with foam was placed into a Blue M™ box-type muffle furnace at 1000°C (+/-)10°C until the foam ignited (~2–8s)[8]. The decomposition of calcium nitrate tetrahydrate into NO₃⁻ and CaO and ammonium phosphate into NH₄⁺ and PO₄³⁻ drives the combustion reaction while the oxidation and complete decomposition of urea energetically sustains the reaction. Reaction 1

2.2 Powder Stoichiometry

The ratio of calcium nitrate tetrahydrate (CaN) to ammonium phosphate (AmP) directly determines the amount of calcium and phosphate in the system [3]. This ratio will be referred to as the calcium to phosphate (c:p) ratio. For example, to obtain a stoichiometric ratio (3:2) of calcium to phosphate in the products, a c:p ratio of 1.5 is needed. Therefore the CaN term (X) in reaction 1 needs to be multiplied by 3 and the AmP term (Y) needs to be multiplied by 2. C:p ratios and the coefficients used to obtain these ratios are shown in Table 1.

A stoichiometric fuel ratio (amount of urea to the amount of CaN and AmP) was determined by calculating an elemental equivalence ratio (ϕ_e = Sum of oxidizing and reducing elements in the mixture) for the reaction [9, 35]. Calculations for ϕ_e for this system are given by Equation 2.

$$3[Ca(NO_3)_2 \cdot 4H_2O] + 2[(NH_4)_2HPO_4] + Z[CO(NH_2)_2] \quad (2)$$

$$3[1]0 + 2[16] + Z[6] = 3$$

Therefore $Z = 3 = \phi_e$.

Table 2 lists the valences for the reactants in this system. These valences were used to calculate ϕ_e .

Fuel ratios were obtained by multiplying (ϕ_e) by the elemental stoichiometric coefficient multipliers shown in Table 2. For example to obtain a stoichiometric fuel ratio, the urea term (Z) in reaction 1 is multiplied by 3.

Table 3a&b present matrices of sample stoichiometries studied. Samples in Table 3a have varying c:p ratios, and a constant fuel ratio (urea:CaN+AmP). Samples in Table 3b have a constant c:p ratio, but varying fuel ratios.

2.3 Product Characterization

Scanning electron microscopy (SEM) was performed with a FEI Quanta 600 SEM. High vacuum with a 20kv beam and spot size of 4.0 created optimal measurement conditions and minimized charging [36]. The CaP powders were sputter-coated with gold (Hummer V) and

analyzed for composition, morphology, grain growth and specific structural formations (i.e. Necking).

X-ray diffraction (XRD) patterns were obtained with a Phillips Analytical PW3240 X'PERT data collector using 2θ scans ranging from 10 to 80 degrees. An internal silicon powder (99.9% pure, Alfa Aesar) with a 1:4 ratio of silicon to CaP was used to correct for machine error [37, 38]. The XRD patterns were corrected with the P' Analytical High Score data program. Peak matching was performed by comparing respective 2θ values to standard peak values in the International Centre for Diffraction Data (ICDD) cards. An error tolerance of (+/−) 0.1 2θ was used to calculate peak values.

A Nicolet 4700 Fourier transform infrared spectrometer (FTIR) (Thermo Electron Corporation) and OMNIC 7.2 software (Thermo Electron Corporation) were used to analyze the molecular structure of the reactants and CaP products. For this work, peak values in the transmittance spectra [10, 12, 28, 29, 39–41] were characterized using peak values from literature [17, 42–44] and the Spectral Database for Organic Compounds (SDBS) provided by the National Institute of Advanced Industrial Science and Technology (AIST).

2.4 Human Fetal Osteoblast 1.19 (hFOB 1.19) Cell Culture

Osteoblasts are responsible for the synthesis of the organic matrix of bone [45, 46]. This line of hFOBs was selected to characterize the response of terminally differentiated cells to the synthesized CaP. Two aliquots of hFOB 1.19 cells (ATCC, CRL-11372), 1–2 million cells/aliquot, were thawed using a double boiler method in a water bath at 37°C for 5min. Each aliquot was transferred to a T-25 TCPS flask (Nunc) and 10ml of cell culture media (37°C); 1:1 Dulbecco's Modified Eagle's Medium/F-12 (HyClone), 10% Fetal Bovine Serum (HyClone) and 0.3mg/ml of G-418 (MP Biomedicals) were added to each flask [47, 48]. The flasks were incubated at 34°C with 98% humidity and 5% CO₂ [47, 48]. This line has been genetically modified for proliferation at 34°C and differentiation at 37°C. Media was changed every 2–3 days [47, 48]. The cells were checked daily and counted weekly. When the flasks reached 80% confluent they were expanded. The media was aspirated off the cells and 2ml of 0.25% trypsin-EDTA (MP Biomedicals) was added. The flasks were incubated for 15min at 37°C with 98% humidity and 5% CO₂. The mixture of trypsin and cells was dispensed into a T-75 TCPS flask (Nunc), supplemented with 25ml of media (37°C) and incubated at 34°C with 98% humidity and 5% CO₂. After reaching 80% confluent the cells were expanded into a T-175 flask (Nunc) and 45ml of cell culture media (37°C) was added [47, 48]. The flasks were incubated at 34°C with 98% humidity and 5% CO₂.

2.5 Cell Culture Experimental Procedure

CaP powders were sterilized in a drying oven (Binder) at 105°C for 24h. After sterilization 100µg of powder was placed in 5 test wells on each tissue culture polystyrene (TCPS) 6-well plate (Fisher). The sixth well was a control and contained only cells. The experiment was run in triplicate. Wells were seeded at a density of $\sim 5 \times 10^3$ [47] and 8ml of cell culture media (37°C) was added to each well. The medium was changed every 2–3 days. During media aspiration the glass pipette did not contact the bottom of the well, minimizing the amount of powder removed from the well plate during media changes. The well plates were incubated

at 39.5°C with 98% humidity and 5% CO₂, examined daily for signs of contamination and tracked microscopically. On days 1, 3, 7, 14 and 21[49] 2ml of media from each well was transferred into a sterile vial and stored at -4°C. Day 1 started 24h after seeding. At the conclusion of the experiment media was aspirated from the wells and the cells were fixed with a 1:0.25 glutaraldehyde (MP Biomedicals) to sodium cacodylate buffer (Electron Microscopy Sciences) solution [49] to stabilize the cell membrane and prevent degradation.

The bottoms of the well plates were cut off (Dremel) and the surface with the fixed cells and CaP was sputter-coated with gold (Hummer V). SEM was performed with a FEI Quanta 600 using high-resolution, 15kv and a spot size of 3.5. Additionally, field emission-SEM (FE-SEM) and energy dispersive spectroscopy (EDS) were performed with a JEOL JSM-7000F SEM to analyze composition, mineralized matrix formation and osteoblast morphology.

CaP bioactivity was tested directly with mammalian cell culture, a more accurate representation of the biologic environment *in vivo* as compared to the oversimplified method of testing in simulated body fluid (SBF) without cells or tissue. The amount of bone alkaline phosphatase (ALP-b) in media collected during co-culture was measured with an ALP-b microassay (Alkaline Phosphatase Detection Kit Fluorescence, Sigma). These measurements were used to track changes in the amount of ALP-b, a direct measure of CaP bioactivity, throughout the experiment and to quantify the effects of c:p ratio and fuel ratio on ALP-b secretion. Readings were taken at 2min intervals for 10min on a Synergy 4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Inc.) and analyzed with the Gen5 data analysis software. Data from the readings taken at 10min are reported. Differences in ALP-b activity were statistically analyzed with Kruskal-Wallis one-way ANOVA ($p < 0.05$) followed by a Mann-Whitney *post hoc* test (Minitab 16, Minitab Inc.) to assess significance ($p < 0.10$).

3.0 Results and Discussion

In previous work it was noted that DCS ignition times were consistently between 2–8s and reaction times were less than 15s from initiation to completion [3, 8]. During reactions, multiple points in the foam ignited at once followed by propagating combustion waves, indicative of simultaneous or explosive combustion [50]. The reactions produced a friable, white, porous powder that upon gentle handling crumbled into finer powder particles. No fluid remained post reaction.

SEM images of powders post-synthesis (Figs. 1a&b) show varied porosity as well as a crystalline microstructure [2, 51]. There appears to be no obvious uniform particle geometry that would indicate sintering of the reactants. Product morphology is indicative of a SHS reaction [2, 7, 52].

Porosity data analysis for this research is presented elsewhere [8]. Briefly, total porosity (P_{Total}) values for sample c:p1.3 x=4.5 and sample c:p1.7 x=4.5 are 57.8 and 52.5%, respectively. The product powder has an average particle size of 52–58µm and as a result it does not meet the suggested minimum porosity and pore diameter required for bone ingrowth [53, 54]. Subsequently, it could not be considered applicable as a bone scaffold but rather as a mineralized, bioactive component in a larger construct e.g. composite bone graft

substitutes [55, 56], implant coatings [56, 57], bone cement [31, 58]. The pore sizes determined from image analysis [8] show pores that could support osteoblast migration [19, 59–61] and the measured porosity increases surface area subsequently increasing adsorption sites for selected proteins [62–64].

3.1 DCS Produced Multiphasic CaP Powders

Figure 2a and Table 4 show XRD patterns where the fuel ratio was held constant at 4.5 and the calcium to phosphate (c:p) ratios were 1.3, 1.5 and 1.7. The CaP powder contains multiple phases. Characteristic α -TCP peaks (ICDD Card 03-0681); β -TCP peaks (03-691, 06-0425); and HA peaks are present (72-1243, 73-0293). Figure 2b and Table 4 show three systems where the fuel ratios were 3, 4.5 and 7.5 while the c:p ratio was held constant at 1.5. Characteristic α -TCP peaks, β -TCP peaks and HA peaks are present. Additionally, carbonate apatite (CA) peaks were identified (011-3826). Carbonate ions are formed from the decomposition of urea [8] during DCS. The carbonate ions could substitute into the CaP structure resulting in carbonite apatite formation [11, 12, 15, 40]. Carbonate concentration in the synthesized DCS products could be increased by increasing fuel ratio or by performing reactions in a CO₂ atmosphere or with the addition of carbonate containing reactants (CaCO₃) similar to traditional methodologies used to incorporate carbonate ions into CaP products [10, 11, 15].

Reaction ignition temperature was 1000°C and maximum reaction temperatures reached up to 1550°C but due to different wave front velocities, reaction rates and cooling rates the reaction temperature at specific areas in the sample could vary between 800 and 1550°C. This range encompasses the formation temperatures on the CaO-P₂O₅ phase diagram [29, 65] for α -TCP, β -TCP and HA peaks. These phases were present in the CaP product XRD patterns, indicating that reaction temperatures throughout the sample fluctuated between 800 and 1550°C.

FTIR was used to characterize product composition and molecular structure. IR peak values correlate to specific bonds. IR spectra of CaP products with varying c:p ratios and constant fuel ratio are shown in Figure 3a and Table 5. A weak OH⁻ peak corresponding to OH stretching vibrations [29, 39, 66] is found in all spectra at peak values between 3437 and 3571 cm⁻¹. A very strong PO₄³⁻ peak corresponding to the anti-symmetric P-O stretching mode [29, 39, 66] is present in all of the systems between 1061 and 1024 cm⁻¹. The second PO₄³⁻ peak between 550 and 600 cm⁻¹ corresponds to the O-P-O bending mode [29, 66]. A very weak P-OH peak (~2160 cm⁻¹) is present in all of spectra. Two small CO₃²⁻ peaks corresponding to the ν_3 stretching vibrations between 1455 and 1411 cm⁻¹ are present in all spectra. These CO₃²⁻ peaks could be correlated to a B-type carbonate ion substitution in the CaP structure [10, 12, 40, 41, 67]. Two very weak CO₃²⁻ peaks are present between 880–800 and 720–680 cm⁻¹ [10, 39, 41, 67, 68]. These CO₃²⁻ peaks could be correlated to either an A- or B-type carbonate ion substitution in the CaP structure [10, 12, 40, 41, 67]. An additional CO₃²⁻ could be present between 1090–1020, but it is masked by the strong PO₄³⁻ peak. A weak NO₃⁻ peak (~1639 cm⁻¹) is present in all CaP spectra. The NO₃⁻ peaks indicate that there was not a complete conversion of reactants to products during the DCS reaction.

IR spectra of CaP products with varying fuel ratios and constant c:p ratios are shown in Figure 3b and Table 5. All spectra, except c:p1.5 x=7.5, contain a very weak OH⁻ peak between 3437 and 3571cm⁻¹. A very strong PO₄³⁻ peak corresponding to the anti-symmetric P-O stretching mode [29, 39, 66] is present in all of the systems between 1061 and 1024cm⁻¹. The second PO₄³⁻ peak between 550 and 600cm⁻¹ corresponds to the O-P-O bending mode [29, 66]. A very weak P-OH peak (~2160 cm⁻¹) is present in all spectra. Two small CO₃²⁻ peaks corresponding to the ν₃ stretching vibrations between 1455 and 1411cm⁻¹ are present in all spectra except c:p1.5 x=7.5. Two very weak CO₃²⁻ peaks are present between 880–800 and 720–680 cm⁻¹ in all spectra. An additional CO₃²⁻ could be present between 1090–1020 but it is masked by the strong PO₄³⁻ peak. A weak NO₃⁻ peak (~1639cm⁻¹) is present in all CaP spectra. A weak NH₂ shoulder peak at 1154cm⁻¹ is present in all spectra except c:p1.5 x=4.5. It is interesting to note the significant increase in intensity of this peak for the c:p1.5 x=7.5 sample. As the fuel ratio increases, the amount of urea available to form NH₂ and CO₃²⁻ increases resulting in increased peak intensity.

Trace amounts of NO₃⁻ are present in the product powder as a result of an incomplete conversion of reactants to products during the combustion reaction. The amount of amine (NH₂) present in the spectra is directly controlled by the fuel ratio (Fig. 3b) and indirectly controlled by the c:p ratio. Sample c:p 1.3 x=4.5 exhibits an NH₂ peak even though it has a fuel ratio of x=4.5. It is speculated that because the c:p ratio is so low the reaction is CaN deficient and it is likely that excess AmP in the reactants reduces the amount of fuel consumed during DCS generating excess urea byproducts (e.g. NH₂, CO₃²⁻).

Reaction 3 could be modified further to incorporate a mixed A/B type carbonate apatite formation.

Where $0 < C < 1$ [12].

3.2 The Synthesized CaP Powders were Not Cytotoxic

Cytotoxicity and cell morphology were analyzed with SEM. Figure 4a shows osteoblasts in the control well at the conclusion of the experiment. The cells appeared to adhere to the well plates and colonized the surface in a uniform monolayer. Figure 4b shows osteoblasts with a flattened morphology attached to the well plate surface and uniformly spreading around the CaP powder (white arrows). Cell adhesion to and proliferation across the CaP could indicate an attempt to colonize the CaP covered surface. Thus suggesting that the CaP materials are surface reactive i.e. they encourage the formation of a physico-chemical bond between the cells and CaP material (24, 25), which could result in bone tissue integration in vivo. Cell morphology in the presence of CaP powder is comparable to areas without CaP particulates [69].

Cell morphology, cellular response and matrix mineralization were observed with FE-SEM. Figure 5a shows cells displaying typical, healthy morphology [70] in the presence of CaP

particulates (white arrows). The cells appeared flattened with pseudopodia extending out from obvious advancing and trailing edges. The cells maintained contact with neighboring cells through multiple projections (Fig. 5b)[69, 71]. The projections (arrow heads) varied in length from 20–100µm.

3.3 CaP Powders Did Not Inhibit Cellular Activity

Spherical nodules (black arrows) developed on the osteoblasts (Figs. 6a&b). EDS (Fig. 7) was used to characterize the elemental composition of the spherical growths. They contained calcium, phosphorous, carbon and oxygen, indicative of matrix vesicle (MV) formation [72, 73]. Sodium and chlorine peaks are present in the EDS spectrum due to the glutaraldehyde/sodium cacodylate fixative applied to all specimens while trace amounts of carbon result from the osteoblasts and TCPS well plates. MVs are cell-derived structures that contain high concentrations of calcium and inorganic phosphate, which creates an environment favorable for the nucleation of apatite crystals [72, 73]. MVs are commonly found *in vivo* at the mineralization front of developing and healing bone and they play a large role in matrix mineralization [72, 73]. The presence of MVs on the osteoblasts indicates that the CaP particulates did not inhibit cellular activity.

3.4 C:p and Fuel Ratio Determine Product Chemistry and Composition and Subsequently Effect Cellular Response and Biologic Activity

The amount of bone specific alkaline phosphatase (ALP-b) present in the osteoblast culture media on days 1, 3, 7, 14 and 21 was measured with an ALP-b microassay. ALP-b is considered the single most accurate marker of bone formation [67, 74] and the secretion of ALP-b is often used to describe osteoblast differentiation [67, 74]. ALP catalyzes the hydrolysis of phosphomonoesters at an alkaline pH, providing inorganic phosphate for crystal formations [72]. Additionally, ALP hydrolyzes pyrophosphate, a strong inhibitor of CaP deposition at the extracellular level [72].

Figure 8a shows ALP-b content for samples with varying c:p ratios and a constant fuel ratio. A significant difference in ALP-b content for all samples ($p < 0.05$) was noted between days 7 and 21 and days 14 and 21 (Fig. 8a). On Day 7 ALP-b content was higher for cells grown on c:p1.3 x=4.5 and c:p1.7 x=4.5 than for c:p1.5 x=4.5 and the control ($p < 0.10$). On day 21 ALP-b content for cells grown on c:p1.5 x=4.5 and 1.7 x=4.5 was higher than the control ($p < 0.10$). Additionally, substrate c:p1.5 x=4.5 resulted in a higher ALP-b content than c:p1.3 x=4.5 ($p < 0.10$). On day 21 the cultures associated with larger c:p ratios (e.g. c:p 1.5, c:p1.7) exhibited increased ALP-b content indicating that CaP powders with these c:p ratios can extend elevated ALP-b secretion levels compared to cell cultures with low c:p ratios.

Figure 8b exhibits ALP-b microassay data for osteoblast cultures grown on substrates prepared with varying fuel ratios and a constant c:p ratio. Similar to Figure 8a a significant difference in ALP-b content for all cultures ($p < 0.05$) was measured between days 7 and 21 and days 14 and 21 (Fig. 8c). On Day 7 ALP-b content for c:p1.5 x=3 and the control was similar ($p > 0.10$) and less than c:p1.5 x=7.5 but greater than c:p1.5 x=4.5 ($p < 0.10$). ALP-b content for c:p1.5 x=7.5 was significantly greater than c:p1.5 x=4.5 ($p < 0.05$). On day 21 ALP-b content for c:p1.5 x=3 was significantly higher than the control ($p < 0.10$). On day 21

the culture grown on the substrate prepared with the lowest fuel ratio (e.g. $x=3$) exhibited increased ALP-b content indicating that CaP powders with low fuel ratios can prolong the period of increased ALP-b secretion levels compared to those from substrates prepared with high fuel ratios.

Previous data show that biologically active CaP materials synthesized with alternative methods can induce a cellular response i.e. MV mediated ALP-b secretion [67, 74] during bone matrix mineralization. Subsequent to the first apatite crystals forming within the MV, ALP-b secretion is down-regulated [67, 74–76]. An advantage of CaP powders synthesized via DCS is that their effects on cellular response and bioactivity *in vitro* can be manipulated by reaction parameters (fuel, c:p ratio). The biologic response they induce can mimic that of the control (cells only) or induce and sustain an extended period of elevated ALP-b secretion (Figs.8a–d) [67, 74], which could enhance early phase biomineralization.

C:p and fuel ratio govern DCS reactions and subsequently dictate product chemistry and composition [8]. The relationship between c:p ratio and ALP-b secretion was not clearly defined in this study, however preliminary data show that DCS combustion temperature is directly related to product composition i.e. the phases (α -, β -TCP, HA, CA) present and the percentage of each phase present. Additional research on the impacts of c:p ratio on combustion temperature and subsequently product composition could elucidate the relationships between c:p ratio and product composition on cellular function and bioactivity. Additionally, the use of diffraction techniques beyond the scope of this research i.e. Rietveld refinement could provide a more detailed examination of the effects of c:p ratio on product phase composition.

Conversely, the relationships between fuel ratio and cellular function and bioactivity were determined. As fuel ratio increased, NH_2 and CO_3^{2-} content in the product powder increased (Fig. 3b). On day 21 the substrate prepared with the lowest fuel ratio (c:p1.5 $x=3$) exhibited the highest cellular ALP-b production (Fig. 8d) indicating that substrates with low fuel ratios (low NH_2 and CO_3^{2-} content) prolong elevated osteoblast ALP-b secretion whereas ALP-b secretion in cultures grown on substrates with high fuel ratios was similar to the control. These results suggest that CaP DCS reaction parameters can be used to directly control *in vitro* biologic activity.

4.0 Conclusions

Multiphasic CaP powders with specific c:p and fuel ratios were produced with a novel decomposition combustion synthesis system. The synthesized powder had a bimodal pore size distribution with an interconnected pore network. Stoichiometric and non-stoichiometric powders were obtained by varying reaction reactant ratios. Controlling reactant ratios generated powders with similar phases. Fuel ratio directly determined NH_2 and CO_3^{2-} content in the final product while c:p ratio indirectly determined NH_2 and CO_3^{2-} content in the final product.

In vitro, hFOB cells cultured with CaP powder displayed a flattened morphology and adhered to and uniformly encompassed the CaP particulates indicating an attempt to colonize the CaP. MV containing calcium and phosphorous were present on the osteoblasts

indicating that the CaP did not down-regulate cellular activity. Reaction parameters (fuel, c:p ratio) can be used to control product composition which influences osteoblast ALP-b secretion. Calcium phosphate biopowders produced via decomposition combustion synthesis are bioactive and exhibit significant biologic potential.

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Fig. 1.

Fig. 1a. SEM micrograph of c:p1.3 x=4.5 powder at 1000x.

Fig. 1b. SEM micrograph of c:p 1.7 x=4.5 powder at 1000x.

Fig. 2.

Fig. 2a. XRD patterns of product powders with varying c:p ratios and a constant fuel ratio.

Fig. 2b. XRD patterns of product powders with varying fuel ratios and a constant c:p ratio.

Fig. 3.

Fig. 3a. Combined IR spectra of product powders with varying calcium to phosphate ratio (c:p1.3 x=4.5, c:p1.5 x=4.5, c:p1.7 x=4.5).

Fig. 3b. Combined IR spectra of product powders with varying fuel ratio (c:p1.5 x=3, c:p1.5 x=4.5, c:p1.5 x=7.5).

Fig. 4.

Fig. 4a. SEM micrograph of osteoblasts in control well at 150x.

Fig. 4b. SEM micrograph of osteoblasts and CaP (white arrows) at 250x.

Fig. 5.

Fig. 5a. FE-SEM micrograph of CaP particulates (white arrows) sample c:p1.5 x=4.5 and cells at 300x.

Fig. 5b. FE-SEM micrograph of CaP particulates (white arrows) sample c:p1.5 x=4.5 and cells with projections (black arrow heads) at 300x.

Fig. 6.

Fig. 6a. FE-SEM micrograph of MV (black arrow) at 1,000x.

Fig. 6b. FE-SEM micrograph of multiple MV on an osteoblast (black arrows) at 2,000x.

Fig. 7.
Spot scan EDS of spherical growths on an osteoblast containing calcium, phosphorous, carbon and oxygen.

Fig. 8.

Fig. 8a. Graph of ALP-b microassay results for varying c:p ratios with a constant fuel ratio. A significant difference in ALP-b content for all osteoblast cultures was noted between days 7 and 21 (■ $p < 0.05$ vs. day 21) and days 14 and 21 (◇ $p < 0.05$ vs. day 21).

Fig. 8b. Graph of ALP-b microassay results for varying fuel ratios with a constant c:p ratio. A significant difference in ALP-b content for all osteoblast cultures was noted between days 7 and 21 (■ $p < 0.05$ vs. day 21) and days 14 and 21 (◇ $p < 0.05$ vs. day 21).

Table 1

C:p ratios with their corresponding coefficients.

c:p Ratio	CaN (X) Coefficients	AmP (Y) Coefficients
1.3	3	2.3
1.5	3	2
1.7	3	1.765

Table 2

Elemental equivalence ratio, φ_e , stoichiometric coefficient multipliers and the calculated urea coefficients that were studied, $\varphi_e = 3$. Valences of elements used in this system.

Elemental Stoichiometric Coefficient Multiplier	Urea (Z) Coefficients	Element	Valence
1 (φ_e)	3	P	+5
		Ca	+2
1.5 (φ_e)	4.5	C	+4
		H	+1
2.5 (φ_e)	7.5	O	-2
		N	0

Table 3

C:p and fuel ratios used for each sample. The stoichiometric ratio for this system is denoted by an*. All other systems are non-stoichiometric.

a. Varying c:p Ratio		Fuel Ratio
1.3		
1.5*		4.5
1.7		
b. Varying Fuel Ratio		c:p Ratio
3*		
4.5		1.5
7.5		

Table 4List of the peaks observed (2θ) in the XRD analysis.

	α -TCP	β -TCP	HA	CA
c:p1.3 x=4.5	14.0	20.4	17.8	33.9
c:p1.5x=4.5	18.5	23.4	28.9	39.4
c:p1.7 x=4.5	19.1	26.2	50.7	40.0
	47.3	32.9	52.1	41.1
		35.2		41.3
		39.3		48.0
x=3 c:p1.5	14.0	16.3	17.9	33.9
x=4.5 c:p1.5	19.1	20.4	28.4	39.4
x=7.5 c:p1.5	24.0	26.4	50.4	40.0
	30.8	32.9	51.9	41.1
	35.1	35.2	53.4	41.3
	47.3	39.3		48.0

Table 5

Results of FTIR analysis. All values correspond to the wave numbers (cm^{-1}) of the different hydroxyl, phosphate, carbonate, nitrate and amine bands.

	OH stretch	PO_4^{3-} anti-symmetric P-O stretch	PO_4^{3-} O-P-O bend	P-OH	CO_3^{2-}	CO_3^{2-}	NO_3^-	NH_2
c:p1.3 x=4.5	3437				1455	880-800		
c:p1.5 x=4.5	3571	1061-1024	550-600	2160	1411	720-680	1639	
c:p1.7 x=4.5			550-600					
x=3 c:p1.5	3437	1061-1024	550-600	2160	1455	880-800	1639	1154
	3571				1411	720-680		
x=4.5 c:p1.5	3437	1061-1024	550-600	2160	1455	880-800	1639	
	3571				1411	720-680		
x=7.5 c:p1.5		1061-1024	550-600	2160		880-800	1639	1154
						720-680		