Surface potential and osteoblast attraction to calcium phosphate compounds is affected by selected alkaline hydrolysis processing

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This study examines the link(s) between the suspension behavior of calcium deficient apatites (CDAs) and biphasic calcium phosphate (BCP), as measured by the ζ-potential, with respect to both whole bone and osteoblasts. CDA is fabricated by hydrolyzing an acidic CaP such as dicalcium diphosphate dihydrate (DCPD; $CaHPO_4 \cdot 2H_2O$) and has a structure and composition close to bone apatite. Sintering CDA results in the formation of BCP ceramics consisting of mixtures of hydroxyapatite (HA) and beta-tricalcium phosphate (β-TCP), with the HA/β-TCP weight ratio proportional to the Ca/P ratio of CDA. The choice of the base for the DCPD hydrolysis allows various ionic partial substitution of the formed CDA. Na for Ca partial substitution is of interest because of the resulting improvement in mechanical properties of the resulting BCP ceramics and NH_4OH was used as a negative control. The ζ-potential was measured for these materials and the stability of the ceramic to bone interaction calculated. ζ-potential values decrease for CDA(NH_4OH) versus CDA(NaOH) and increase for BCP(NH_4OH) versus BCP(NaOH). While results of these analyses indicate that NH_4OH and NaOH processed CDA and BCP will likely yield osteoblast attachment *in vivo*, differences in the ζ-potentials may explain varying degrees of cell attachment.

Introduction

The surface potential of orthopedic biomaterials in contact with bone has been the subject of recent interest, however, the exact mechanisms of the implant to bone bond are not clearly understood [1–3]. While surface functional groups such as carboxyl and hydroxyl ions determine the surface charge and hence the surface potential of many common ceramics, the degree to which such surface groups alter the resulting ceramic to osteoblast or bone attachment is less understood [4, 5]. Bioceramics such as hydroxyapatite (HA; $Ca_{10}(PO_4)_6(OH)_2$) and beta-tricalcium phosphate (β-TCP; $Ca_3(PO_4)_2$), have been a focus in the development of osteoconductive scaffolds for bone tissue engineering and improved orthopaedic implant coatings [6–8]. They respectively present hexagonal and rhombohedral crystal symmetries [9].

The link between the surface potential, as assessed by the zeta (ζ) potential, of these bioceramics and their resulting attraction to both bone and osteoblasts, have been shown in previous studies [1–3]. The focus of this paper is the relationship between modifications in the processing method of calcium phosphate (CaP) powders and their resulting ζ-potential, and hence suitability for use as bone tissue engineering scaffolds. Several studies have linked the surface, or ζ-potential of bone to the piezoelectric effect. Oppermann *et al*. [2] examined the stability of the interactions between bone and various compositions of Bioglass™ and HA using ζ-potential data as a function of pH. Their results showed that unstable interactions were indicative of successful *in vivo* bonding between optimum Bioglass™ compositions and bone and between HA and bone. An effective method to quantitatively examine bone-implant bonding is to measure the mobility of colloidal particles in a suspension, using ultrasonic attenuation spectroscopy (UAS), from which the surface, or ζ-potential is calculated. This study will employ ζ-potential analysis using UAS, as a function of physiologically relevant pH, to predict bone-implant interactions in a variety of bioceramic powder materials that have been synthesized via two different processing methods.

One possible way of forming calcium deficient apatite (CDA) with both a structure and composition close to bone apatite is by hydrolyzing an acidic CaP phase such as dicalcium diphosphate dihydrate (DCPD; $CaHPO_4 \cdot 2H_2O$) [10, 11]. Sintering CDA between 900 and 1200 °C results in the formation of biphasic calcium

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phosphate (BCP) ceramics consisting of mixtures of HA and β-TCP with the HA/β-TCP weight ratio proportional to the Ca/P ratio of CDA [12]. The choice of the base for the DCPD hydrolysis allows various ionic partial substitution of the formed CDA. Na for Ca partial substitution is of interest because of the resulting improvement in mechanical properties of the final BCP ceramics [13]. Although biological fluids contain large quantities of ionic sodium, the biological role of ionic sodium within CaP materials, with respect to cell adhesion and activity, remains unclear. In order to measure the influence of Na-substitution on the surface potentials of CaP materials, two kinds of chemical bases NH_4OH and NaOH were chosen, knowing that NH_4OH does not induce any particular ionic substitution.

The degree of ionic activity between bone and synthetic materials may be assessed through the ζ-potential as calculated from the acoustophoretic particle mobility. O'Brien [14, 15] and Oja [16] have extensively described the electrokinetic phenomena of acoustophoresis. The ζ-potential is calculated using the acoustophoretic mobility with a correction for the particle inertia $G(\alpha)^{-1}$, in an alternating field. This correction reduces the velocity amplitude of particle motion and was derived using the Helmholtz–Smoluchowski equation [14, 15].

$$\zeta = (\mu\eta/\varepsilon_o\varepsilon_r)G(\alpha)^{-1} \tag{1}$$

where ε_o is the permitivity in a vacuum, ε_r is the relative permitivity, and $G(\alpha)^{-1}$ is given by:

$$G(\alpha)^{-1} = \left[1 - \frac{i\alpha(3 + 2\Delta\rho/\rho)}{9\left\{1 + (1-i)(\alpha/2)^{1/2}\right\}}\right] \tag{2}$$

To describe the effects of homo- and heterocoagulation and to generate a quantitative theory for the overall kinetic stability of a system of non-identical particles, Hogg *et al*. [17] developed an expression for interparticle stability (W_{ij}). This factor, termed the stability ratio is in effect, the ratio of particle collisions to collisions that result in agglomeration.

$$W_{ij} = \int_{a_i + a_j}^{\infty} \exp\left(\frac{V_T}{kT}\right)\frac{dr}{r^2} \tag{3}$$

where the interparticle intercomponent stability (W_{ij}) varies exponentially with the total force, V_T, (attractive and repulsive) and inversely with the square of the particle separation distance, r, from a separation equal to the sum of the two particle radii, a_i and a_j to an infinite separation distance into the solution. The repulsive force, which acts as a barrier to coagulation is proportional to the surface charge of the particle. However, this surface charge is not readily measurable because of continuous ion activity at the particle/fluid interface. Therefore, the surface charge is estimated at the double layer by the ζ-potential, which in this study, is measured using UAS methods. Stable interactions, i.e., suspensions for which there is no flocculation exist at $W > 20$, while unstable interactions are predicted for systems where $W < 20$.

Materials and methods

Powder processing methods

Calcium deficient apatite ($Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$) powders were obtained by hydrolyzing two 40 g batches of DCPD powder (Merck, Darmstadt, Germany) in aqueous alkaline solutions (500 ml with [NaOH] = 0.35 M and [NH_4OH]= 0.3 M). These two chemical reactions were performed at 90 °C under stable magnetic stirring for 4 h. The CDA powders were washed with distilled water and dried (80 °C, 12 h). They were then calcinated at 1050 °C for 4 h to obtain a BCP, a solid mixture of HA and β-TCP.

Powder analysis

The purity of the starting and final materials was analyzed by assessing specific surface area (SSA) and Na content: SSA of both CDA and BCP powders were measured by BET adsorption and the sodium content was checked using atomic absorption spectroscopy.

Suspension preparation

In this study, the O'Brien model, which takes into effect double-layer distortion, was used to calculate the ζ-potential from the UAS mobility data, which was measured using the AcoustoSizer II ESA apparatus (Colloidal Dynamics, Warwick, RI). To eliminate adsorption of ions from the atmosphere, all ζ-potential measurements for each of the materials and cells examined in this study were carried out under a N_2 blanket. Measurements were not made in the presence of an adsorbed protein such as bovine serum albumin as prior work has shown it to have only minimal effect on the surface potential [18]. All suspensions were prepared in physiologic saline (PS; 0.154 M NaCl) at a concentration of $\sim$ 1 vol %.

Bone

Bone stock was taken from the proximal femur of a deer harvested within 4 h of death and sectioned for immediate grinding. The soft tissue and periosteum were removed and the bone wet ground from the subperiosteal surface to the mid-cortex, while submerged in PS, using a diamond conical grinding bit at 3000 rpm, under moderate hand pressure. The resulting bone particle suspension was then subjected to immediate ζ-potential and particle size measurement.

Osteoblasts

MC3T3-E1 mouse osteoblast cells were cultured in α-minimum essential medium (Invitrogen Corp., Carlsbad, CA) supplemented with 10% foetal bovine serum (Atlanta Biologicals, Norcross, GA) at 37 °C in a humid atmosphere containing 5% CO_2 and 95% air. The media was aspirated off and the cells washed once with 1X phosphate buffered saline. Cells were then trypsinized and subsequently pelleted by centrifuging at 10 000 rpm for 10 min. The resulting OB pellets were resuspended by aspiration in PS in 50 ml tubes. PS was prepared at three different pH levels (7.3, 7.4, 7.5) by

titrating with complementary acid and base (1 M HCl and 1 M NaOH). The resulting suspensions were then transferred to 60 ml syringes and finally, injected in turn into the UAS cell chamber where instantaneous mobility measurements were taken and ζ-potential values calculated and recorded as a function of pH. This technique is known as static ζ-potential measurement (as the suspension does not constantly flow through the chamber) and was chosen because of the small volume of OB suspension available for measurement.

Bioceramics

Two types of CDA (hydrolyzed using NaOH and NH_4OH) along with two types of BCP bioceramic were assessed for differences in their ζ-potential. The average diameters of the CDA (NaOH), CDA (NH_4OH), BCP (NaOH) and BCP (NH_4OH) were measured, under identical suspension conditions, as 0.95, 0.85, 1.0 and 0.93 μm, respectively. Because the magnitude of the ζ-potential is indirectly proportional to the diameter of the particulate material, the bone was ground until a particle size of 1 μm was reached. These particle sizes were also measured as a function of pH to detect agglomeration and each of the powders remained essentially unagglomerated throughout the pH range examined. Each powder was suspended in PS and subjected to ζ-potential measurement with the AcoustoSizer II under a $-N_2$ blanket. The ζ-potential was then collected and plotted as a function of physiologically relevant pH. The pH range was controlled with automated titration with complementary acid (1 M HCl) and base (1 M NaOH) under constant stirring.

Results and discussion

Powder analysis

FTIR spectra (not shown) verified that the classic PO_4 and OH vibration peaks were present and that the presence of carbonate and hydrogenophosphate bands was minimally present for both CDA powders [19].

Rietvelt analysis is applied to XRD data for polycrystalline materials in order to better refine peaks, yielding a more accurate determination of the crystal structure. In this study, Rietvelt refinement was used to determine the lattice parameters of the HA and β-TCP in BCP(NaOH) (HA: $a = b = 0.9416(2)$; $c = 0.6887(2)$/ β-TCP: $a = b = 1.0408(2)$; $c = 3.7228(9)$) and in BCP(NH_4OH) (HA: $a = b = 0.9420(2)$; $c = 0.6881(2)$/ β-TCP: $a = b = 1.0403(2)$; $c = 3.7368(9)$) (all values in nanometers). The HA/β-TCP weight ratio was found to be $3.48/6.52 \pm 0.301$ for BCP(NH_4OH) and $3.53/6.57 \pm 0.352$ for BCP(NaOH). This yielded the atomic Ca/P ratios for both CDA/BCP(NH_4OH): 1.56 and CDA/BCP(NaOH): 1.57. Only the c values for β-TCP, present in both BCP powders, were significantly different. This indicates that Na for Ca substitution occurs preferentially in the β-TCP phase. According to the crystal structure of β-TCP, the only possible Ca site is the incomplete one designated as Ca(4).

Specific surface area was determined using BET adsorption. The SSA is $120.3 \pm 0.15\,m^2/g$ for CDA(NH_4OH), $60.7 \pm 0.05\,m^2/g$ for CDA(NaOH) and $2.85 \pm 0.01\,m^2/g$ for both BCP powders. Atomic absorption spectroscopy measurements give Na content for both CDA(NaOH) and BCP(NaOH) as $1.97 \pm 0.09\%$. For both CDA(NH_4OH) and BCP(NH_4OH), the amount of Na was found to be $0.45 \pm 0.09\%$. This latter result is due to Na impurities present in the DCPD ($CaHPO_4 \cdot (H_2O)$), which is used as a starting material in the alkaline hydrolysis.

ζ-potential

ζ-potential data, as a function of pH, for the CaP powders is shown in Fig. 1. The iso-electric point (iep) is the pH at which the net surface potential is zero. The iep for the CDA powders varies from a pH of 6.8 for the CDA (NH_4OH) shifting to a more basic pH of 7.4 for the CDA (NaOH), while the BCP processed with NH_4OH does not exhibit an iep over the pH range tested and the BCP processed using NaOH has an iep at pH 6.4. The BCP (NH_4OH) powder behaves very much like commercially available HA, having relatively little positive potential and no iep [18]. The ζ-potential values range from 5.1 to -0.7 mV for the CDA (NaOH), 1.5 to -4.2 mV for the CDA (NH_4OH), 1.5 to -2.8 mV for the BCP (NaOH) and 2.9 to 1.5 mV for the BCP (NH_4OH) over the approximate pH range from 6 to 8. Sodium substitution in the CDA powders caused a slight increase in the overall ζ-potential curve while in the BCP powders, it led to a slight decrease in the overall ζ-potential values. This decrease in the BCP ζ-potential with sodium ion substitution is logical considering that Na ions having a $+1$ charge are substituting for Ca ions having a $+2$ charge, thus decreasing the overall net charge within the material. The smaller positive shift in the CDA materials may indicate either that an insufficient amount of Na was substituted into the structure and/or that NH_4OH caused other changes in the surface chemistry of CDA that resulted in the noted increase in the overall surface potential.

Kowalchuk *et al*., [18] using bone particles having a diameter of > 5 μm, reported ζ-potential values from 0 to -15 mV, while the bone particles used in this study have a diameter of 1 μm and a relatively constant ζ-potential of -75 mV across comparable pH ranges [20]. Because the magnitude of the ζ-potential is indirectly proportional to the diameter of the particulate material, the ζ-potential values obtained in this study are therefore consistent with this earlier finding [3, 14, 21–23].

Stability

Stability calculations plotted as a function of pH for CDA(NaOH), CDA(NH_4OH), BCP(NaOH) and BCP(NH_4OH) are shown in Figs. 2–5. At all pHs tested, the CDA(NaOH) remained unstable ($W_{11} < 20$) and is predicted to flocculate in suspension in physiologic saline while the bone shows values of stability, $W_{22} < 20$, and is hence stable over all test pHs. Interestingly, even though there is a difference in both the sign and hence the magnitude of the ζ-potential between the bone and the CDA(NaOH), the interaction between the bone and bioceramic (W_{12}) is predicted to be stable ($W_{12} > 20$) and hence each component will

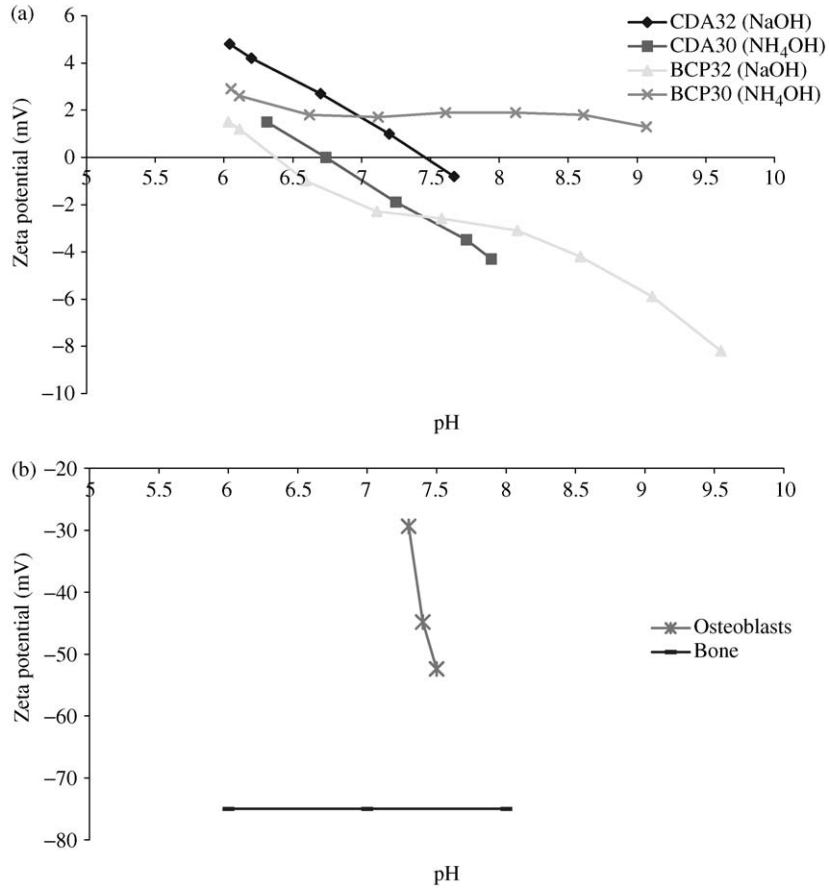

Figure 1 ζ-potential for CDA(NaOH), CDA(NH_4OH), BCP(NaOH), and BCP(NH_4OH) (a). ζ-potential for MC3T3-E1 mouse osteoblasts and ground bone (b). Both (a) and (b) are shown as a function of physiologically relevant pH at solids loadings of $\sim$ 1 vol % in 0.154 M NaCl electrolyte.

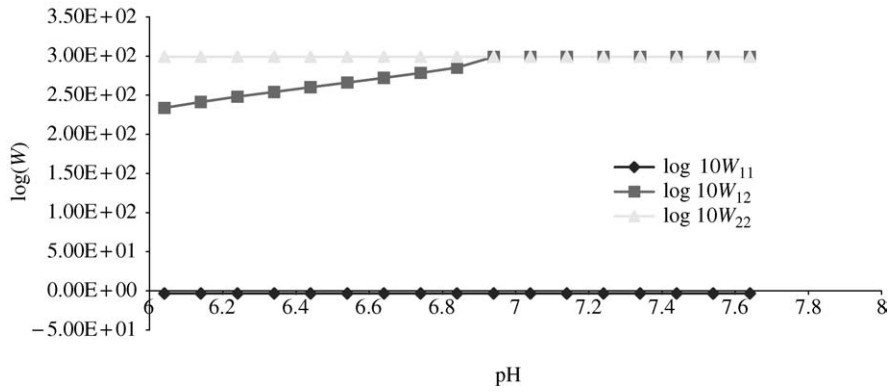

Figure 2 Stability calculations for CDA(NaOH), denoted W_{11}, bone (W_{22}) and the interaction between CDA(NaOH) and bone (W_{12}).

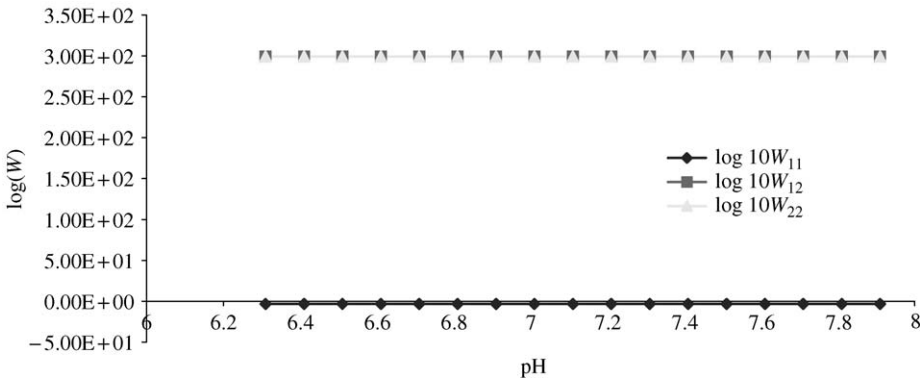

Figure 3 Stability calculations for CDA(NH_4OH), denoted W_{11}, bone (W_{22}) and the interaction between CDA(NH_4OH) and bone (W_{12}).

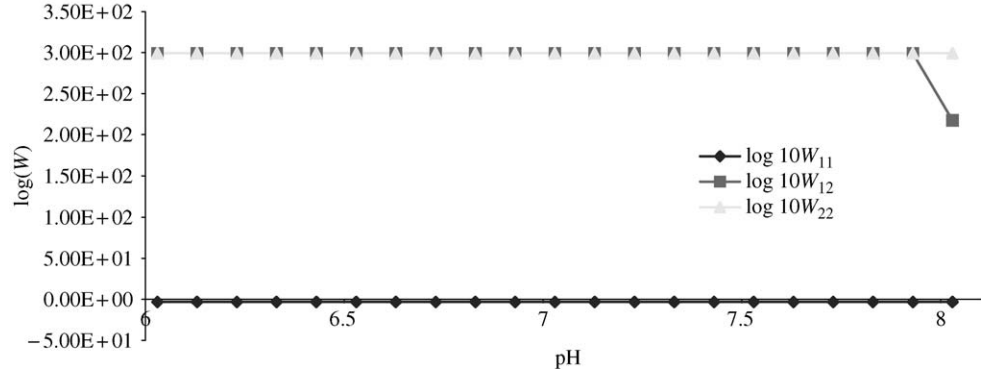

Figure 4. Stability calculations for BCP(NaOH), denoted W_{11}, bone (W_{22}) and the interaction between BCP(NaOH) and bone (W_{12}).

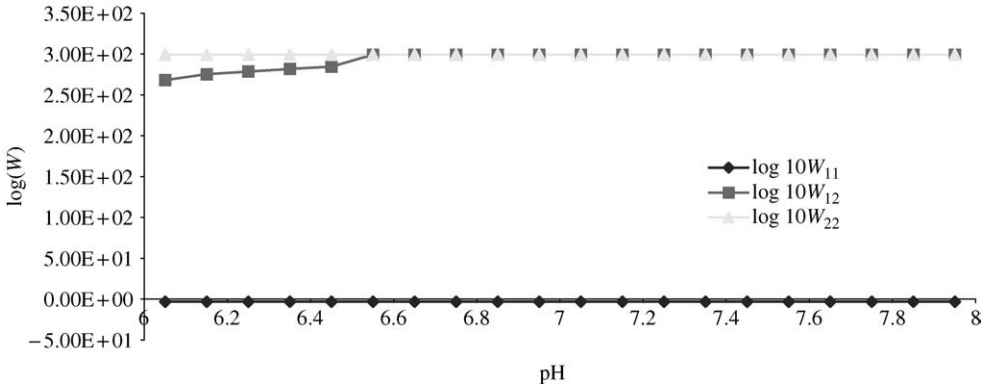

Figure 5. Stability calculations for BCP(NH4OH), denoted W_{11}, bone (W_{22}) and the interaction between BCP(NH4OH) and bone (W_{12}).

remain separately stable and unflocculated over the pH range. Similar results were also found for the CDA(NH_4OH), BCP(NaOH) and BCP(NH_4OH).

While it is well known that osteoblasts attach to CaP surfaces, and indeed bone has been shown to integrate into these implant materials, the mechanisms for such bonding are not completely understood [24]. The ζ-potential results in this paper agree with those reported by Ducheyne *et al*. who found small ζ-potentials of 0–10 mV using $< 5\,\mu m$ HA powders [25]. The powders in this study were sub-micron in size, giving comparable magnitudes and sign for the ζ-potential. Using a comparably sized HA powder (diameter $< 5\,\mu m$) purchased from a commercial supplier, Oppermann *et al*. [1] report much greater ζ-potentials on the order of ~ 130 mV. Therefore, while both of these powders are nominally HA, they have very different surface potentials, which are likely to result from differences in powder processing and surface area.

So, while the HA-based powders in this study have similar particle sizes and ζ-potential magnitudes, the effect of different processing treatments, such as sodium substitution on both the surface potential and SSA, must still be further evaluated. As each of the powders examined in the current study are CaP-based, it is logical to expect that their surface potentials are similar, which indeed, the data from Fig. 1 illustrate. The primary difference between the two groups of CaP powders is the nature of the inorganic base (NH_4OH and NaOH) in the hydrolysis reaction during powder processing. Sodium ions are added to substitute partially for Ca in both the HA and β-TCP phases, where the Ca/P ratio decreases with Na substitution but the (Ca + Na/2)/P ratio remains constant, and these Na ions will then alter the surface chemistry. Such cation substitutions, if concentrated on the powder surface, should yield a disproportionate change the net overall surface potential, and hence the resulting ζ-potential of the powder. However, the SSA was found to be twice as high for (CDANH_4OH), at $\sim 120\,m^2/g$, in comparison to CDA (NaOH), at $\sim 60\,m^2/g$. This doubling of SSA with NH_4OH may also be a factor in understanding why sodium substitution did not produce the expected decrease in the ζ-potential for the CDA powders. In addition, if it is assumed that only the atoms at the surface of the CaP are contributing to the surface potential, then a 2% addition of Na to the particle only involves $\sim 1.3 \times 10^{-4}$% increase in Na at the surface of 1 μm particles. Therefore, such a slight change may not be detected by ζ-potential analysis. Further work will include measuring the ζ-potential of CDA and BCP powders with increased Na substitution to verify the nature of the changes in the surface potential.

Differences in the ζ-potential in turn necessitate changes in the experimental protocol if these powders are processed in aqueous slurries. For example, the greater the magnitude of the ζ-potential, the easier it is to disperse and form a uniform slurry. A uniform slurry is necessary to form a uniform green body, and hence a uniform scaffold [26]. None of the ceramic materials tested in this study have a sufficiently large enough ζ-potential to exist as a dispersed, stable suspension, however all the CaP powders examined have ζ-potential curves that have a sufficiently large enough electro-negativity gap with respect to both bone and osteoblasts

to expect that each CaP would favor the initial attachment of osteoblasts.

Such differences may be the key to interpreting conflicting osteoblast attachment results. Given the significant difference between the magnitude and sign between the ζ-potentials of each of the CaP powders and both the osteoblasts and bone at pH 7.4 (≥ 50 mV and ≥ 70 mV, respectively), it may be that while the stability curves rightly represent the thermodynamic steady-state, osteoblast attachment to any of the CaP powders examined in this paper is more accurately characterized by differences in the sign of the net ζ-potentials at a given pH because osteoblast attachment occurs over short times, involving multiple organic and cellular components.

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