Osteoblast responses to as-deposited and heat treated sputtered CaP surfaces

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The clinical success of dental implants is governed by implant surfaces and bone cell responses that promote rapid osseointegration. The objective of this study was to evaluate the *in vitro* osteoblast cell response to heat treated and non-heat treated CaP coatings. In this study, the heat treated surfaces exhibited a poorly crystallized HA-type structure whereas the non-heat treated surface exhibited an amorphous structure. The heat treated CaP surfaces were observed to have a mean contact angle measurement of 57.95 ± 0.95 degrees, whereas the non-heat treated CaP surfaces were observed to have a mean contact angle measurement of 44.6 ± 0.3 degrees. From the *in vitro* cell culture study, the ATTC CRL 1486 human embryonic palatal mesenchyme (HEPM) cells displayed a similar protein production and hexosaminidase activity on the heat treated and non-heat treated CaP surfaces throughout the nine day experiment. However, the HEPM cells cultured on non-heat treated CaP surfaces were observed to have higher specific ALP activity after nine days' incubation compared to cells cultured on heat treated CaP surfaces. The higher specific ALP activity by cells on non-heat treated surfaces were suggested to be attributed to the lower degree of crystallinity and the lower contact angles observed in this study.
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1. Introduction

Plasma-sprayed hydroxyapatite (HA) and calcium phosphate (CaP) coatings on titanium (Ti) implants are used to improve initial osseointegration. However, numerous problems with the plasma-sprayed HA coatings have been cited, including variations in bond strength between the coating-metal interface, non-uniformity in coating density, alterations in HA structure as a result of the process, and poor adhesion between the coating-metal interface [1–4]. In addition to the crystallographic and chemical variations and poor metal-coating adhesion, the coating qualities of plasma-sprayed HA utilized in most studies in the literature are either unknown, poorly known, or left unstated [5]. The unknown coating quality has led to many conflicting animal and clinical observations.

It has been suggested that amorphous CaP coatings will exhibit enhanced osteoblast activity compared to crystalline CaP coatings. The rationale for this hypothesis stems from the mechanisms underlying the phenomena of bioactivity. Increasing dissolution of the implant surfaces due to low crystallinity produces solution mediated events affecting cellular activity, organic matrix deposition, or mineral precipitation [6, 7]. However, it has been reported that amorphous coatings have an adverse effect on the establishment of an interface with bone, whereas in other studies, the amorphous coatings were reported to be advantageous if coating longevity is desired [8, 9].

Lacefield *et al*. concluded that sputter coating may be the method of choice for coating HA onto implants [10]. Although surface and bulk characterization of these sputtered coatings has been previously reported by us and other investigators in the literature [11–18], the biological response to sputtered CaP coatings has not been fully understood. In addition, it has also been reported that the as-sputtered coatings were amorphous and that crystallized HA-type coatings were produced after heat treatments above 500 °C [14, 17]. Since it has been reported that CaP surfaces such as HA coated implants improve osseointegration, the objective of this study is to investigate an optimum CaP surface for maximum bone response. In this study, the *in vitro* activities of osteoblast precursor cells in response to heat treated sputtered CaP coatings were evaluated.

2. Materials and methods

2.1. Sputter Coating

Commercially pure titanium disks (Ti) of 1/8 inches thick × 0.6 inches diameter (Munford, AL) were ground sequentially from 240 grit to 600 grit. The ground Ti disks were then ultrasonically cleaned using Alconox

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detergent (Alconox, NY) for 30 min, followed by rinsing with deionized water and ethanol. Prior to sputtering, the disks were plasma cleaned for 2 min using Plasma-Spreen II-973 system (Plasmatic Systems, Inc, NJ). The disks were then placed in the RF sputtering NCR 3117 system (Vacuum Technology Associates, CO) and the chamber was pumped down to a base pressure of 6×10^{-6} torr. High purity argon (99.999%) was back-filled into the chamber, bringing the pressure to about 10^{-4} torr. At an energy of 300 watts and a RF voltage of 1000 V, CaP coatings were produced using a plasma-sprayed HA target (Ca/P ratio of 1.6). At a rate of 0.2 μm per hour, a coating thickness of 1.4 μm was achieved after 7 h sputtering. The coatings were then divided into two groups; non-heat treated coatings (control) and heat treated coatings. The heat treated coatings were subjected to a post deposition heat treatment of 700 °C for 90 min.

2.2. X-ray diffraction (XRD)

XRD analysis was performed to evaluate the structure of the CaP coatings. A Siemens D500 diffractometer using Cu K_α radiating having energies of 40 keV and 30 mA was used. The incident X-rays passed through 3° and 1° slits before impinging upon the CaP coatings. Diffracted X-rays passed through 1°, 0.6° and 0.05° slits at the X-ray counter. Three samples for each treatment were analyzed and the data were collected from 25° to 35° 2θ at 0.1° per minute scan rate. Crystalline coatings were identified by matching the peaks with standard synthetic HA (JCPDS 9-0432). The crystallite size of the coatings was calculated based on the 002 reflections using Scherrer equation.

2.3. Contact angle

In order to evaluate the surface energy of the CaP coatings, the wettability of CaP surfaces were measured using a video contact angle VCA-2000 system (Advance Surface Technologies, MA). Double distilled water was used as a medium for measurement. Five CaP surfaces from each treatment were used. At an α value of 0.05, the surface energies of CaP coatings were statistically analyzed using ANOVA, with differences assessed using post-hoc pair wise comparisons of individual group means using the Fisher's Protected Least Significant Difference test.

2.4. Cell Culture study

The cell culture study was conducted using the ATCC CRL 1486 human embryonic palatal mesenchyme cells (HEPM), an osteoblast precursor cell line, over a 9 day period. The CaP surfaces were seeded with 15 000 cells/ml in Dulbecco's Modified Eagle's Medium (DMEM) containing 7% fetal bovine serum, 1% antibiotic-antimycotic solution, and 50 μg/ml ascorbic acid. One ml of the cell suspension was plated per well of a 24 well plate containing a CaP sample. The cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. At confluency, the media was removed and replaced with DMEM containing 2% fetal bovine serum, 1% antibiotic-antimycotic solution, 50 μg/ml ascorbic acid, and 4 mM β-glycerophosphate. The culture medium was changed every two days with DMEM media containing 2% fetal bovine serum, 1% antibiotic-antimycotic solution, 50 μg/ml ascorbic acid, and 4 mM β-glycerophosphate. On days 3, 6 and 9, cells on four heat treated and non-heat treated CaP surfaces were lysed using triton 0.2% solution after decanting the media and washing twice with a phosphate buffered solution then stored at − 20 °C.

2.5. Cell Number Assay

Cell number was determined by measuring the hexosaminidase concentration. On the day of the assay 50 ul of each cell suspension dilution was added to wells in a flat bottom plate. A solution of 40 ul of substrate buffer was used. The substrate consisted of 0.0684 g of p-nitrophenyl N-acetyl-B-D-glucosamide, p-nitrophenyl 2-acetamido-2-deoxy-B-D glucopyranoside and 10 ml of a 0.1 M citrate buffer (0.2 M citric acid stock, 0.2 M trisodium citrate dihydrate stock and 40 ml deionized water with a pH of 5.0). The solutions were incubated at 37 °C for 1 h. After 1 h 80 ul of stop buffer was added consisting of 0.05 M glycine 5 mm EDTA with a pH of 10.5 and water was used. The plates were then read using a microplate reader at 405 nm. The number of cells was then determined by correlating the absorbance to a cell number standard curve. The cell number were statistically compared using the ANOVA test ($p < 0.05$).

2.6. Total Protein Assay

Four samples from each group were analyzed for total protein synthesis. Protein synthesis was performed using the Pierce BCA protein assay (Pierce, IL). The cell layer suspension (30 μl) was added to 200 μl of working reagent (sodium carbonate, sodium bicarbonate, BCA detection reagent, sodium tartrate in 0.1 M NaOH, and 4% copper sulfate). The samples were then incubated for 30 min at 37 °C and read using a microplate reader at 600 nm. The absorbance for the cell layer suspension was correlated to a standard protein curve and differences in protein synthesis were statistically compared using the ANOVA test ($p < 0.05$).

2.7. Specific Alkaline phosphatase (ALP) activity

Four samples from each treatment were used for measuring the specific ALP activity. The cell layer suspension (50 μl) was added to 50 μl of working reagent (1.5 M 2-amino-2 methyl-1-propanol, 20 mM p-nitrophenol phosphate, and 1 MM magnesium chloride). The samples were then incubated for 3 h at 37 °C. After 3 h incubation, the reaction was stopped with the addition of 100 μl of 1 N NaOH and read using a microplate reader at 410 nm. The absorbance for the cell layer suspension was correlated to a standard ALP activity curve prepared using p-nitrophenol stock standard. Specific ALP activity of cells cultured on CaP surfaces were then calculated by normalizing the ALP activity to proteins synthesized.

Differences in specific ALP activity was statistically compared using the ANOVA test ($p < 0.05$).

3. Results

3.1. X-ray diffraction (XRD)

No peaks were observed on the non-heat treated CaP coatings, suggesting an amorphous structure. As shown in Fig. 1, a post-deposition heat treatment of 700 °C revealed poorly crystallized coatings with major peak positions matching for HA. The average crystallite size ($\pm$ 1 standard deviation) for the heat treated coatings were observed to be 53.4 ± 0.2 nm.

3.2. Wettability

Using water as a medium for measurement, the non-heat treated CaP surfaces was observed to have a statistically lower contact angle as compared to the heat treated CaP surfaces. The contact angles on the heat treated CaP surfaces and non-heat treated CaP surfaces were 57.95 ± 0.95 and 44.6 ± 0.3, respectively.

3.3. Cell Numbers

As observed in Fig. 2, the number of cells increases over time on CaP surfaces. However, no significant difference in hexosaminidase activity was observed between the heat treated and non-heat treated CaP surfaces.

3.4. Protein

As shown in Fig. 3, no significant difference in the concentration of protein synthesized was observed between the heat treated and the control non-heat treated CaP coatings. However, an increase in protein production over time on CaP surfaces was observed.

3.5. Specific Alkaline Phosphatase Activity

As shown in Fig. 4, no statistical difference in specific ALP activity was observed between the heat treated and the control non-heat treated CaP surfaces on days 3 and 6. However, after day 9 incubation, the specific ALP activity for cells cultured on non-heat treated CaP surfaces was significantly higher than cells cultured on heat treated CaP surfaces.

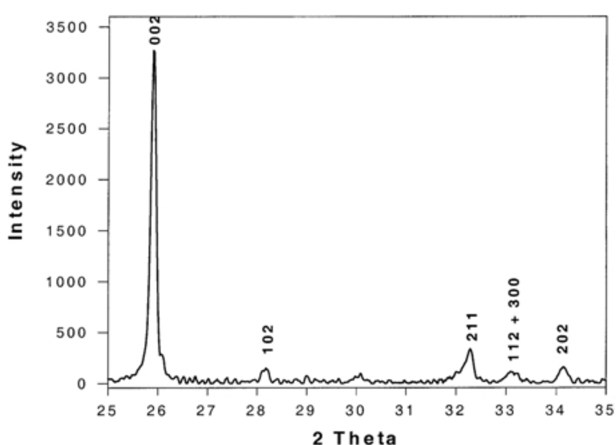

Figure 1 X-ray diffraction of the heat treated CaP surface, showing data collected from 25° to 35° 2Θ.

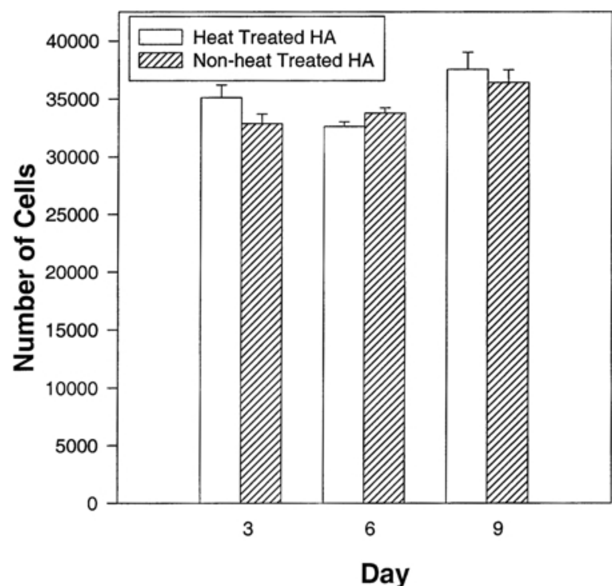

Figure 2 Number of HEPM cells on heat treated and non-heat treated CaP surfaces over time. (Error bar represents 1 standard error.)

4. Discussion

Depending on the properties of biomaterials, different rates of cellular responses have been observed *in vitro* [19–22]. These differences have been attributed to varying surface chemistries and crystallinities. In this study, X-ray diffraction indicated a crystallite size of 53.4 ± 0.2 nm for the heat treated CaP surfaces. Crystallite size of about 500 nm have been reported for HA powders [23, 24]. These differences in crystallite size have been associated with varying degree of dissolution rates, with smaller, more imperfect crystals being subject to greater dissolution [25–27]. However, other studies have suggested that the dissolution of the coatings may

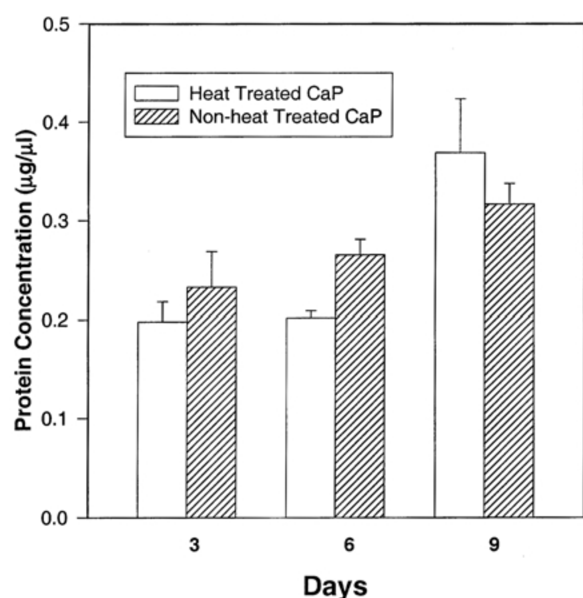

Figure 3 Cell layer and matrix associated protein synthesis by HEPM cells on heat treated and non-heat treated CaP surfaces. (Error bar represents 1 standard error.)

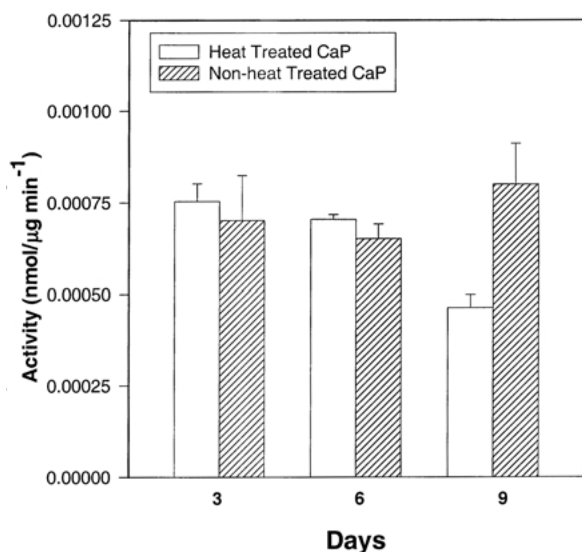

Figure 4 ALP specific activity of HEPM cells on heat treated and non-heat treated CaP surfaces. (Error bar represents 1 standard error.)

result in the supersaturation of calcium and phosphate ions in the physiological media, therefore resulting in the reprecipitation of a crystallized coatings [12, 28, 29]. Evidence of recrystallization was observed when the crystallite size increased after immersion in a physiological solution [12].

Significant difference in wettability between the heat treated and non-heat treated CaP coatings was observed in this study. This difference in wettability property between the two surfaces may be attributed to other surface properties, such as surface roughness and variation in composition as a result of post-deposition heat treatments.

In *in vitro* cell culture studies, protein synthesis is an important marker for evaluating cell function. Matrix proteins in bone have been reported to play a crucial role in the calcification and architectural construction of these hard tissues [30]. In this study, no statistical difference in total cell surface and matrix associated protein was observed between HEPM cells cultured on heat treated CaP surfaces, non-heat treated CaP surfaces, and control tissue culture plastics. No statistical difference in the cell number was also observed between the heat treated and non-heat treated surface, indicating no difference in cell proliferation on CaP surfaces. However, as seen in Fig. 2, the number of cells cultured on CaP surfaces continued to increase over time suggesting continuous cell proliferation with time. Other studies have indicated that although no significant differences in cell numbers were observed, differences in the extra cellular matrix were reported thus inducing apatitic formation [31]. Since it has been observed by many investigators that tissue responds to HA of different crystallinity, these results suggested a certain degree of crystallinity, outside of the range of crystallinity studied in this study, is required in order to elicit differences in protein production and hexosaminidase activity.

Alkaline phosphatase (ALP) specific activity is the other biochemical marker that is commonly used as a marker for determining osteoblast phenotype and is considered to be an important factor in determining bone mineralization [32–35]. Cells grown on the CaP surfaces were observed to exhibit no significant difference in specific ALP activity after 3 and 6 days incubation, indicating a similar rate of cellular differentiation on both surfaces. However, after day 9 incubation, the specific ALP activity for cells cultured on control non-heat treated surfaces was significantly higher than cells cultured on heat treated surfaces. The higher specific ALP activity by cells cultured on control non-heat treated CaP surfaces may be attributed to many factors, such as the lower degree of crystallinity and the lower contact angles observed in this study. As suggested in other studies, differences in cellular differentiation on both the control non-heat treated and heat treated CaP surfaces could also be attributed to the ingestion of CaP particles and intracellular solubilization [36, 37]. It was observed that intracellular dissolution of calcium containing crystals greatly influence cell behavior [38–40]. The presence of CaP particles and its intracellular solubilization were hypothesized to affect calcium and phosphate homoeostatic mechanisms and to modify the mechanical regulators of DNA synthesis without any expression of cytotoxic effect [41]. However, from this study, differences in osteoblast-like phenotype on CaP surfaces of different treatments were indicated, suggesting the need for better characterization of CaP coatings prior to animal and clinical implantation.

5. Summary

As indicated by X-ray diffraction, the heat treated surfaces exhibited a poorly crystallized HA-type structure whereas the control non-heat treated surface exhibited an amorphous structure. Differences in wettability measurements were also observed for heat treated and non-heat treated CaP surfaces. From the *in vitro* cell culture study, the HEPM cells displayed a similar protein production and hexosaminidase activity on the heat treated and non-heat treated CaP surfaces throughout the 9 day experiment. However, from the specific ALP activity, the HEPM cells cultured on control non-heat treated CaP surfaces were observed to differentiate at a higher rate after 9 days' incubation compared to cells cultured on heat treated CaP surfaces. It was suggested from this study that the differences in ALP specific activity by cells on non-heat treated surfaces after 9 days may be attributed to the lower degree of crystallinity and the lower contact angles observed in this study. However, further *in vitro* and *in vivo* studies on these coatings are needed to confirm and understand the effect of heat treatments on host tissues.

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