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Research article

Human Mesenchymal Cell Attachment, Growth and Biomineralization on Calcium-enriched Titania-polyester Coatings

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Abstract: Titanium implant osseointegration can be enhanced by surface modifications that include hydroxyapatite from $Ca_3(PO_4)_2$. However, CaO may provide more surface calcium (w/w) to induce cellular responses. Therefore, the purpose of this study was to compare responses to novel CaO and $Ca_3(PO_4)_2$-enriched titania-polyester (PPC) nanocomposite coatings, which were created by an electrostatic ultrafine dry powder coating technique. EDX confirmed the presence of a base polymer scaffold, biocompatible titanium, and CaO or $Ca_3(PO_4)_2$. SEM showed that human embryonic palatal mesenchymal cells (ATCC CRL-1486) had attached and spread out onto all surfaces within 24 hours. Cell attachment assays showed that there was a progressive increase in cell numbers with surface CaO incorporation (0–5%), such that the PPC + 5% CaO coatings supported the most cells. Furthermore, the PPC + 5% CaO had significantly more ($P = 0.006$) cells attached to their surfaces than the PPC + 5% CaP coatings and titanium controls, at 24 hours. The PPC + 5% CaO also had more cells that had proliferated on their surfaces over 72 hours, although these differences were not significant ($P > 0.05$). Similarly,

MTT assays showed that the cells had sustained metabolic activity on all surfaces. Again, metabolic activities were highest on the PPC + 5% CaO, and they were significantly higher ($P < 0.05$) on all CaO-enriched surfaces (1/3/5% CaO) than on the PPC + 5% CaP. Subsequently, Alizarin Red-S staining detected the initiation of biomineralization within 2 weeks, and abundant mineral deposits after 4 weeks of growth on PPC + 5% CaO and PPC + 3% CaO. These nanocomposite coatings have shown that CaO enrichments may provide a heightened cell response when compared to conventional hydroxyapatite.

Keywords: implants; coatings; titanium dioxide; calcium; mesenchymal cells

1. Introduction

There has been a growing demand for dental implants and orthopedic prostheses to replace diseased, damaged and missing tissues. Accordingly, dental implants and orthopedic prostheses made of commercially pure titanium (cpTi) and titanium alloys (Ti_6AlV_4) respectively, have been widely studied to maximize clinical success. Their immediate and long-term retention and function are dependent on intimate interactions between the biomaterial surfaces and surrounding tissues, in a process known as osseointegration. Implant surfaces that mimic the surrounding extracellular bone matrix may promote favorable cellular responses, which enhance osseointegration. Therefore strategies were developed to enhance titanium surfaces by incorporating chemical inserts such as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), calcium phosphate ($Ca_3(PO_4)_2$) and various ceramics [1–8].

Since hydroxyapatite is the naturally occurring inorganic component in bone and teeth, it has been incorporated onto implant surfaces to enhance protein adsorption, cell attachment, proliferation, differentiation and biomineralization [9]. However, hydroxyapatite surface coatings are usually made by the plasma spray technique [10,11], which creates thick layers that lack surface homogeneity and have low bond strength [12]. They can also be made by sputter coating techniques [13,14], but these are costly and time consuming. Furthermore, hydroxyapatite alone mimics only the inorganic mineral, and lacks the collagenous matrix that is present in teeth and bones. Therefore, polymer/ceramic hybrid coatings have now been developed to combine organic polymers with inorganic minerals [15]. The polymer provides a continuous scaffold with design flexibility [16], and the ceramic inserts promote bone formation [17].

Accordingly, we developed novel polymer/ceramic composite coatings that contain polymeric scaffolds and ceramic inserts [18]. They were created by a novel, simple and inexpensive electrostatic ultrafine dry powder coating technique [19,20]. This method generates uniform, continuous, homogenous and highly adherent polymer coatings with intricate nano-topographies, surface roughness

and a high degree of biocompatibility [21–23]. We augmented their formulation by including small amounts (5% w/w) of commercially available mineral trioxide aggregates (ProRoot® MTA), which are a ceramic-like mixture of mineral oxides ($(CaO)_3 \cdot SiO_2$ + $(CaO)_2 \cdot SiO_2$ + $(CaO)_3 \cdot Al_2O_3$ + $(CaO)_4 \cdot Al_2O_3 \cdot Fe_2O_3$ + $CaSO_4 \cdot 2H_2O$ + Bi_2O_3) [19,24]. Now, we have refined this formulation and shown that either calcium oxide (CaO) or calcium phosphate ($Ca_3(PO_4)_2$) alone can be readily incorporated into their surfaces, without compromising the composition and properties of the base coating [25].

The calcium in these surface coatings may be particularly beneficial, since calcium can promote bone formation around implants. Indeed, a recent study showed that merely the pretreatment of their surfaces with calcium ion containing solutions, promoted bone formation around implants in rabbit femora [26]. Other studies have shown that $Ca_3(PO_4)_2$ coated titanium implants have enhanced bone formation in rat femora [27], promoted the healing of intrabony defects in dog mandibles [28], and increased their resistance to dislodgement in rabbit tibia [29]. Furthermore, silica-$Ca_3(PO_4)_2$ nanocomposite coatings increased the alkaline phosphatase activity of bone marrow mesenchymal stem cells that were attached to their surfaces [30].

These cellular responses are often studied in mesenchymal stem cells that are the undifferentiated precursors to bone forming osteoblasts. Several studies have used a human embryonic palatal mesenchymal cell line (HEPM, ATCC CRL1486) that was derived from the developing palate of a human fetus, which has provided a clinically relevant model to study the cellular response to implant surfaces [31–33]. We found that the human mesenchymal cells attached, spread out, proliferated and differentiated on the surfaces of polymeric coatings, and initiated biomineralization in extended culture [21–23]. Furthermore, these cells responded favorably to the polymer/ceramic composite coatings [18], and to the CaO- and $Ca_3(PO_4)_2$-enriched coatings that were recently created in our refined formulation [25]. However, despite widespread study of $Ca_3(PO_4)_2$ coatings on implants, the cellular response to CaO-enriched surfaces is largely unknown. The CaO additives may in fact deliver more calcium (w/w), although $Ca_3(PO_4)_2$ provides both calcium and phosphorous in a hydroxyapatite-like layer. Therefore, the purpose of this study was to examine the response of human mesenchymal cells to the CaO-enriched coatings, and to compare them to the $Ca_3(PO_4)_2$-enriched coatings, unmodified surface coatings and titanium controls.

2. Materials and Methods

2.1. Formulation of Ultrafine Powders

The ultrafine powders were prepared as previously described (Figure 1) [18,21–23,25]. This involved the use of a base powder formulation that was then enriched with bioactive ingredients (Table 1).

To prepare the base powder, commercially available White Avalanche polyester (LinksCoating, London, Canada) that contained micron-sized TiO_2 (25% w/w) was enriched with small amounts (0.5% w/w) of nano-sized TiO_2 ($nTiO_2$; Degussa, USA) that served as a flow additive to prevent agglomeration of the ultrafine particles [20]. These base powders were then enriched with either CaO (Sigma-Aldrich, Oakville, Ontario) at progressively higher concentrations (1, 3 or 5% w/w), or $Ca_3(PO_4)_2$ (Sigma-Aldrich, Oakville, Ontario) at only the higher concentration (5% w/w), to serve as bioactive agents. All these powders were combined in a high-shear mixer and passed through a sieve (35 μm) to obtain ultrafine particles. Their ultrafine dimensions were then verified through a particle size analysis that was performed by a BT-9300s Laser Particle Analyser (Ningbo Yinzhou Hybers, China). The laser beam interacted with particles suspended in water to create a diffraction pattern that was then analyzed to determine particle size.

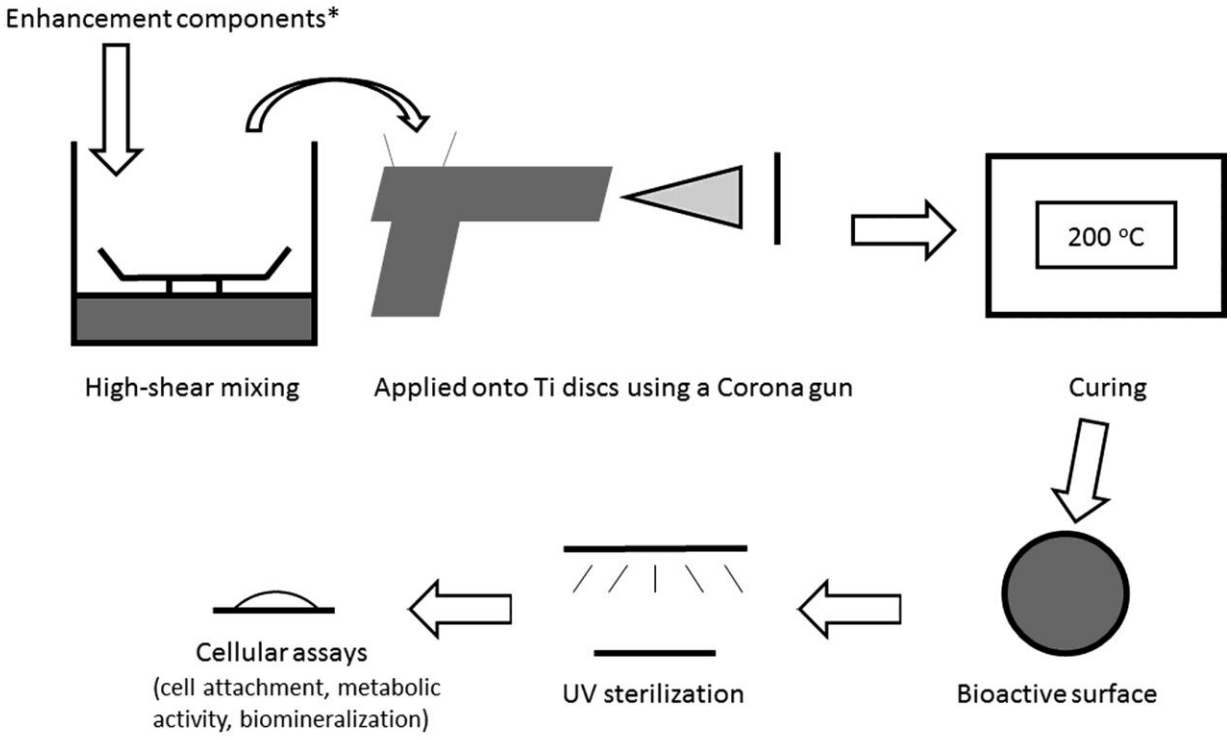

Figure 1. Schematic overview of coating technique and analysis. An ultrafine dry powder coating technique was used to create novel biocompatible surface coatings enriched with bioactive agents, which were then evaluated in cellular assays. Polyester resin, micron-sized titanium dioxide (TiO_2), nanoparticles of titanium dioxide ($nTiO_2$), and functional additives that included calcium oxide (CaO) or calcium phosphate ($Ca_3(PO_4)_2$), were ground and mixed in a high-shear mixer to create ultrafine particles. The ultrafine powders were then sprayed onto commercially pure titanium (cpTi) surfaces and cured in a furnace. The surface coatings were sterilized and used as substrates in cellular assays.

Table 1. Coating Formulations and their Controls.

Experimental Groups	*Surface Nomenclature*	*Bioactive Agent*	*Flow Modifier*	*Biocompatible Agent*	*Polymeric Scaffold*	*Metal Substrate*
Substrate Control	Ti	-	-	-	-	cpTi [c]
Coating Control	PPC	-	$nTiO_2$ (0.5%) [a]	TiO_2 (25%) [a]	Polyester Resin [b]	
CaO	PPC + 1% CaO	CaO (1%) [a]				
	PPC + 3% CaO	CaO (3%) [a]				
	PPC + 5% CaO	CaO (5%) [a]				
$Ca_3(PO_4)_2$	PPC + 5%CaP	$Ca_3(PO_4)_2$ (5%) [a]				

[a] Formulations based on weight percent (w/w); [b] Avalanche-White polyester resin; [c] Commercially pure titanium (cpTi).

2.2. Preparation of Surface Coatings

Surface coatings were created with the formulations, by using an ultrafine dry powder coating technique [19,20], as previously described [18,21–23,25] (Figure 1). The base powder formulation (PPC), CaO-enriched PPC (PPC + 1%CaO, PPC + 3%CaO, PPC + 5%CaO) and $Ca_3(PO_4)_2$-enriched PPC (PPC + 5%$CaPO_4$) were sprayed onto sheets of cpTi (Grade 2, thickness = 0.5 mm, McMaster-Carr, Cleveland, OH). They were sprayed with a Corona Gun (Nordson, Westlake, OH) at a set voltage (20 kV), so that the ultrafine particles became ionized and electrostatically attracted to grounded cpTi. The coated surfaces were then cured (200^{o}C, 10 minutes) in a high performance air flow oven (Sheldon Manufacturing, Inc., Cornelius, OR). The PPC-coated cpTi sheets were then cut into circular disks (diameter 24 mm) for analysis.

To confirm their composition, the surface coatings were chemically analyzed as previously described [18,23,25]. The coated titanium disks were mounted onto metal stubs, secured with adhesive carbon tape and sputter coated with nano-sized gold particles (10 nm). They were then analyzed by energy dispersive X-ray spectroscopy (EDX) using a Hitachi S-4000 SEM (Hitachi, Pleasonton, CA) with a working voltage (15 kV) and distance (15 mm). The EDX analyses were repeated at multiple separate locations on each surface, and a representative chromatogram selected.

2.3. Cell Culture Studies

The surface coatings and their controls were prepared to serve as the substrate layer in cell culture studies. The coated and uncoated titanium disks were rinsed (x3) with phosphate buffered saline (PBS, Gibco, pH 7.4, calcium chloride and magnesium chloride free), and then rinsed (x3) with trypsin (Gibco, 0.25% with EDTA and phenol red). They were then placed in polypropylene tubes (BD Falcon, 50 ml), submerged in fresh trypsin (0.25%) and sonicated (60 minutes), and then submerged in sodium hypochlorite (2.5%) and sonicated (60 minutes). Following each sonication, the surfaces were washed (x10) with ddH_2O and then washed (x2) with autoclaved ddH_2O. Finally, the surfaces were rinsed (x3) with ethanol (70%) and PBS, and sterilized by UV light (30 minutes on each side) in a tissue culture hood.

The coated and uncoated titanium disks were then placed individually within the wells of 6-well tissue culture plates (BD Falcon) with the prepared surfaces on top to serve as substrates for cell culture. They were covered with Dulbecco's modified eagles medium (DMEM, Gibco) supplemented with fetal bovine serum (10% FBS, Gibco), L-glutamine (2 μmol/ml), penicillin G (100 U/ml), streptomycin sulfate (100 μg/ml) and amphotericin B (0.25 μg/ml). Human embryonic palatal mesenchymal cells (HEPM, ATCC CRL-1486) were seeded onto the center of the discs within the tissue culture plates, and incubated (37^oC, 5% CO_2) with culture media that was replenished every 3 days.

2.4. Cell Morphology and Surface Interactions

Cell morphology and surface interactions with the coatings and their controls were examined by scanning electron microscopy (SEM). The cell cultures on coated and uncoated titanium disks were harvested 24 hours after seeding. The culture media was carefully removed by suction and the surfaces gently rinsed (x3) with PBS. They were fixed (20 minutes) with glutaraldehyde (2.5%, Sigma-Aldrich) in sodium cacodylate trihydrate buffer (100 mM, Sigma-Aldrich) and rinsed (x2) with PBS. Finally they were dehydrated by placement (10 minutes each) in progressively higher concentrations of ethanol (25, 50, 75, 95 and 100%), immersed in hexamethyldisilazane (20 seconds), and air dried at room temperature. The coated and uncoated titanium disks were then mounted onto metal stubs, secured with conductive carbon tape and sputter coated with nano-sized gold particles (10 nm). Their surfaces were examined with a Hitachi S-2600 SEM (Hitachi, Pleasonton, CA).

2.5. Cell Attachment and Growth

The cell attachment and growth on surface coatings and their controls were measured in cell attachment assays. Cell seeding densities were higher (50,000 cells/disk or 11,000 cells/cm^2) for a 24

hour assay, to ensure sufficient numbers of attached cells on all surfaces, and much lower (10,000 cells/disk or 2,200 cells/cm^2) for a 72 hour assay, so that rates of proliferation could be compared. The cell cultures on coated and uncoated titanium disks were harvested 24 and 72 hours after seeding. The culture media, detached and dead cells were carefully removed by suction and the surfaces gently rinsed (x2) with PBS. The disks were then transferred individually to wells in fresh 6-well tissue culture plates and rinsed (x2) again with PBS. Cells that remained attached to the surfaces were released by a trypsin (300 µl/well) incubation (37^{o}C, 5 minutes), mixed with DMEM (300 µl/well), and counted in a hemocytometer.

2.6. Mitochondrial Enzyme Activity

The metabolic activity of cells grown on surface coatings and their controls were measured by MTT assay. The cell cultures on coated and uncoated titanium disks were harvested 24 hours after seeding. The culture media was carefully removed by suction and replaced with fresh media (2 ml/well) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent solution (300 µl/well). Following 4 hours of incubation (37^{o}C), MTT solubilizing agent (1 ml/well) was added and an aliquot of the mixture in each well transferred to a 96-well plate. Their absorbance (570 nm) readings were measured in a Safire Multi-Detection Microplate Reader (Tecan, San Francisco, CA).

2.7. Biomineralization of Cultures

The biomineralization of cultures on surface coatings and their controls was detected by Alizarin Red-S staining. The cell cultures on coated and uncoated titanium disks were harvested 2 and 4 weeks after seeding. The culture media was carefully removed by suction and the surfaces gently rinsed (x2) with PBS. They were fixed (1 hour) in formalin (4%) and rinsed with ddH_2O (calcium-free). The calcified mineral deposits in the cultures were stained (10 minutes) with Alizarin Red-S (2%, EMD) and rinsed with ddH_2O (calcium-free).

2.8. Data Analyses

Cell counts from the attachment assays and absorbance levels from the MTT assay were analyzed by SigmaPlot 12.0 (Systat Software Inc., Chicago, IL, USA). Their mean values and standard errors were calculated, charted and statistically analyzed. Differences were identified by one-way ANOVA, and post hoc comparisons were performed by the Holm-Sidak method with the significance level set at $P = 0.05$.

3. Results

3.1. Bioactive surface coatings were created

The surface coatings were created by an ultrafine dry powder coating technique and enriched with bioactive agents (Figure 1). They all had a very similar chemical composition when analyzed by EDX (Figure 2). All surfaces contained an abundance of carbon (C), oxygen (O) and titanium (Ti). Additionally, those coatings that had been prepared with enriched ultrafine powder formulations contained small amounts of the bioactive agents within their surfaces. The PPC+1%CaO, PPC+3%CaO and PPC+5%CaO coatings contained progressively higher levels of calcium (Ca), which reflected the progressively higher weight ratios of CaO that had been added to their formulations. Similarly, the PPC+5%$CaPO_4$ coating contained small quantities of both calcium (Ca) and phosphorous (P), whereas the PPC coating control had none of these bioactive additives.

3.2. Surface coatings supported cell attachment and spreading

The surface coatings supported human mesenchymal cell attachment and spreading on their surfaces. All coatings had cells that had attached and spread out onto their surfaces within 24 hours of seeding, when viewed by SEM (Figure 3). The cells had attached and spread out onto the coatings as they had on the titanium controls. They were firmly attached, with cytoplasmic extensions and projections onto the underlying surfaces. There were wide and spreading cellular morphologies on all CaO- and $Ca_3(PO_4)_2$-enriched surfaces, and an elongated appearance on the PPC coating control.

3.3. Calcium-enrichment promoted cell attachment

The calcium-enrichment of the coatings enhanced the initial human mesenchymal cell attachment to their surfaces. All CaO-enriched coatings had more cells that attached to their surfaces within 24 hours, than the titanium controls, when counted in cell attachment assays (Figure 4). Furthermore, the progressive increase in CaO enrichment from the PPC + 1% CaO to PPC + 3% CaO and then PPC + 5% CaO coatings was reflected in a progressive increase in the cells counted on their surfaces. These differences were almost significant ($P = 0.06$) and a clear trend was evident so that ultimately the PPC + 5% CaO coatings had the most cells.

The PPC + 5% CaO coatings had many more cells attach within 24 hours, than any of the other surfaces, when counted in cell attachment assays (Figure 5). They had significantly ($P = 0.006$) more human mesenchymal cells that attached to their surfaces within 24 hours, than the PPC + 5% $CaPO_4$ coatings or the PPC and titanium controls.

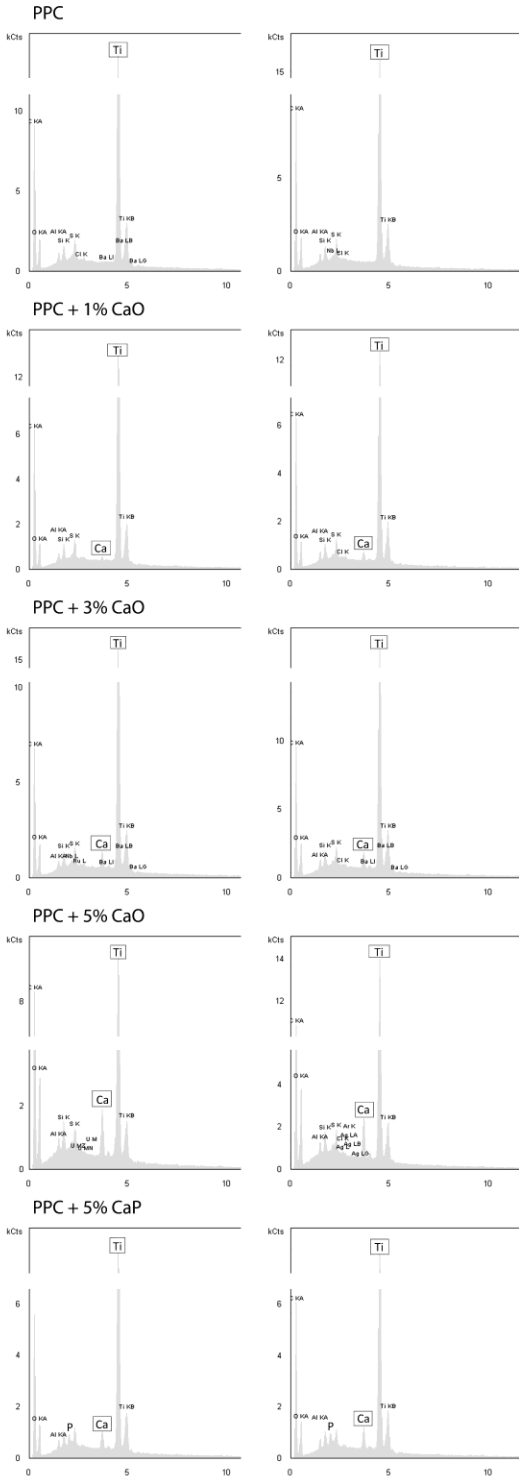

Figure 2. Coatings were analyzed by energy dispersive x-ray spectroscopy (EDX). Titanium (Ti) was detected at high levels in all coatings. Calcium (Ca) was detected at much lower levels in only the PPC+1%CaO, PPC+3%CaO, PPC+5%CaO and PPC+5%CaP surfaces, and in proportion to their formulation. Phosphorus (P) was detected at very low levels in only the PPC + 5% CaP surfaces.

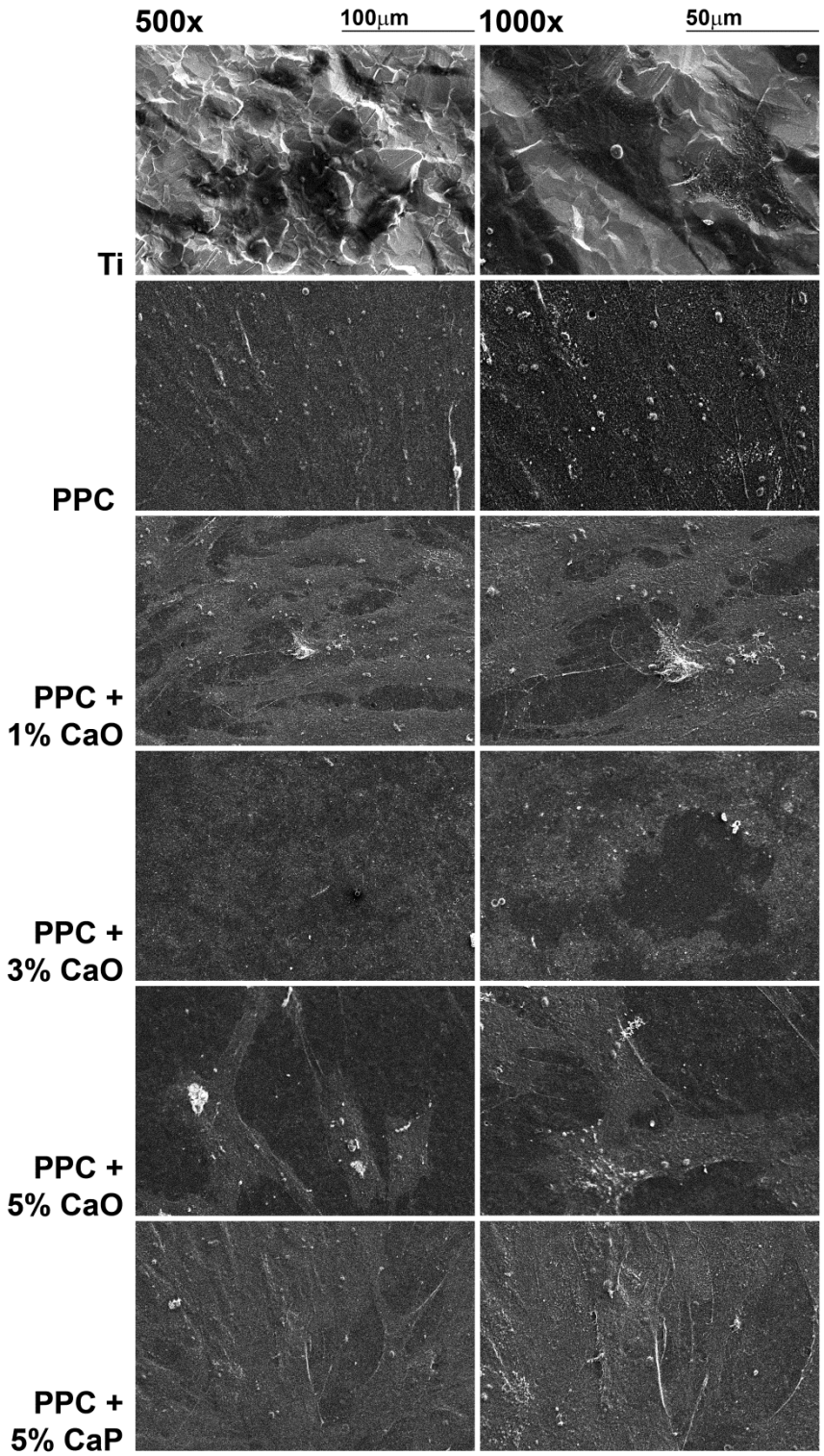

Figure 3. Cell attachment and spreading was examined by scanning electron microscopy (SEM). Within 24 hours of seeding, human mesenchymal cells had attached and spread out onto all coatings. There were elongated cells with spreading morphologies that were tightly attached to the coating surfaces.

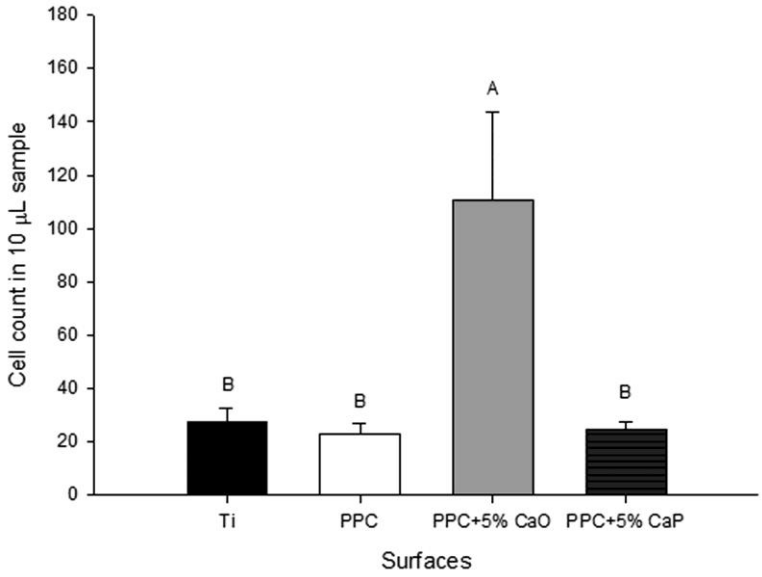

Figure 4. Cells attached to surfaces were counted. After 24 hours, cells that had attached and spread out onto the surfaces were collected and counted. There were five times as many cells on the PPC+5%CaO coatings than on any other surface.

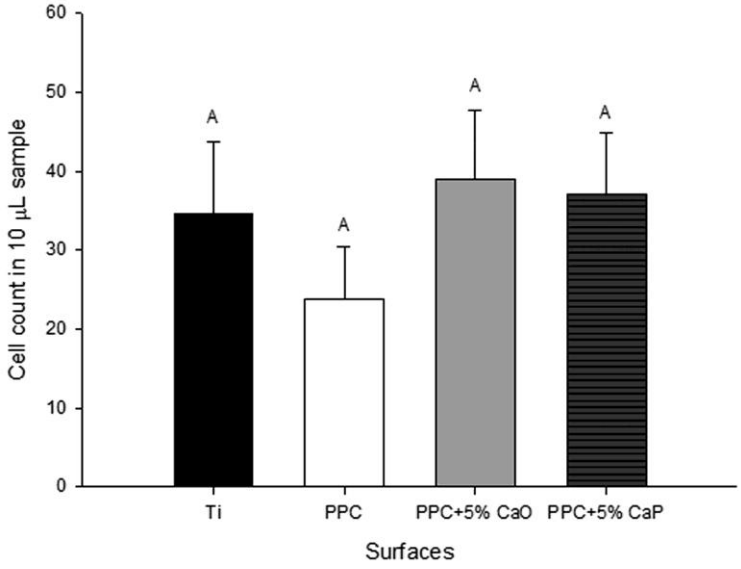

Figure 5. Cell proliferation on coatings were compared. After 72 hours, the cells had attached, spread out and proliferated on the surfaces. Cell numbers were highest on the PPC+5%CaO coatings and lowest on the PPC controls, but mostly similar on all surfaces.

3.4. Cell proliferation and metabolism were sustained

All coatings supported human mesenchymal cell proliferation on their surfaces. All coatings and their controls had more cells growing on their surfaces after 72 hours, than in the first 24 hours, when counted in cell attachment assays (Figure 6). The PPC + 5% CaO coatings had the most cells on their surfaces, followed by the PPC + 5% $CaPO_4$ and titanium, while the PPC control coatings had the least. However, these differences were not statistically significant ($P > 0.05$).

All coatings supported human mesenchymal cell metabolism on their surfaces. All coatings and their control surfaces had cell cultures with active mitochondria, when measured in MTT assays (Figure 7). The CaO-enriched coatings had the most metabolically active cultures that had significantly ($P < 0.05$) higher activity than the PPC + 5% $CaPO_4$ coatings, or the PPC and titanium controls. However, the differences between the PPC + 3% CaO coating and titanium controls were not significant ($P > 0.05$).

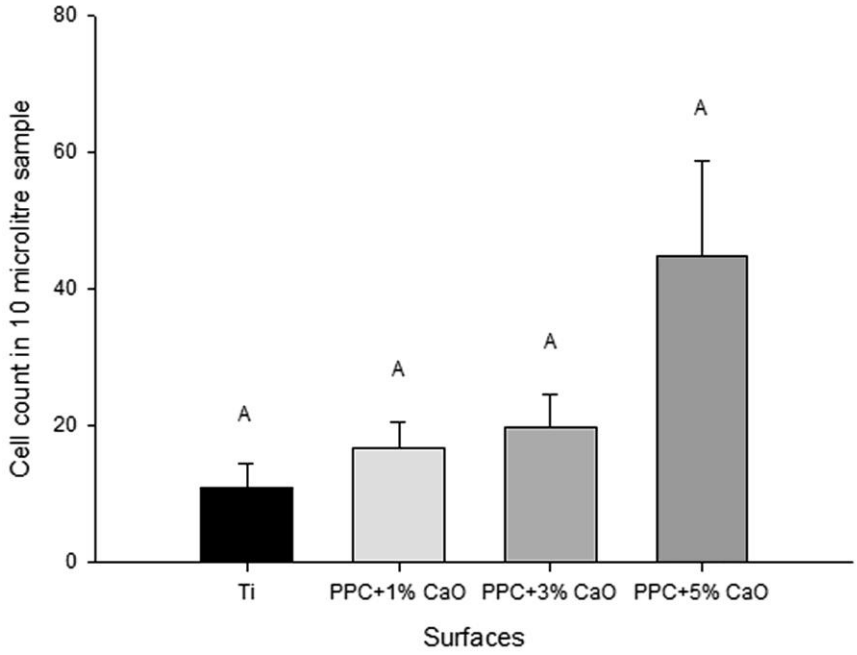

Figure 6. Cells attached to CaO-enriched coatings were counted. Within 24 hours, cell numbers were highest on the PPC+5%CaO coatings, followed by the PPC+3%CaO coatings, the PPC+1%CaO coatings and then the titanium (Ti) controls. There were more than twice as many cells on PPC+5%CaO coatings, than any other surface.

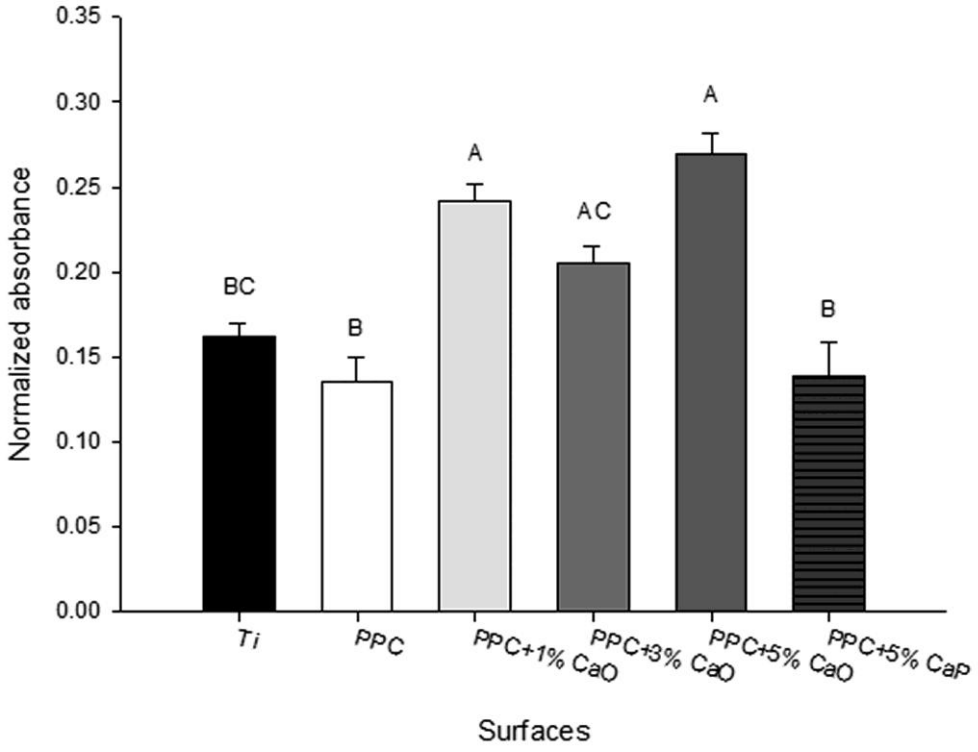

Figure 7. Cell metabolic activities (MTT) were measured. After 24 hours of cell attachment and spreading, they remained metabolically active. Their activities were higher on the PPC + 1% CaO, PPC + 3% CaO and PPC + 5% CaO coatings, than on the PPC+5%CaP, PPC or titanium (Ti) surfaces.

3.5. High-calcium coatings promoted biomineralization

Higher levels of calcium enrichment in the coatings promoted the initiation of biomineralization in human mesenchymal cell cultures growing on their surfaces. The PPC + 3% CaO and PPC + 5% CaO coatings showed numerous mineral deposits in their cell cultures within 2 weeks, when stained with Alizarin Red-S (Figure 8). Similarly, they showed widespread mineralization in their cultures after 4 weeks of growth. However, the PPC + 1% CaO and the PPC + 5% $CaPO_4$ coatings, as well as the PPC and titanium controls had no mineral deposits after 2 weeks, and only sparse mineralization after 4 weeks of growth.

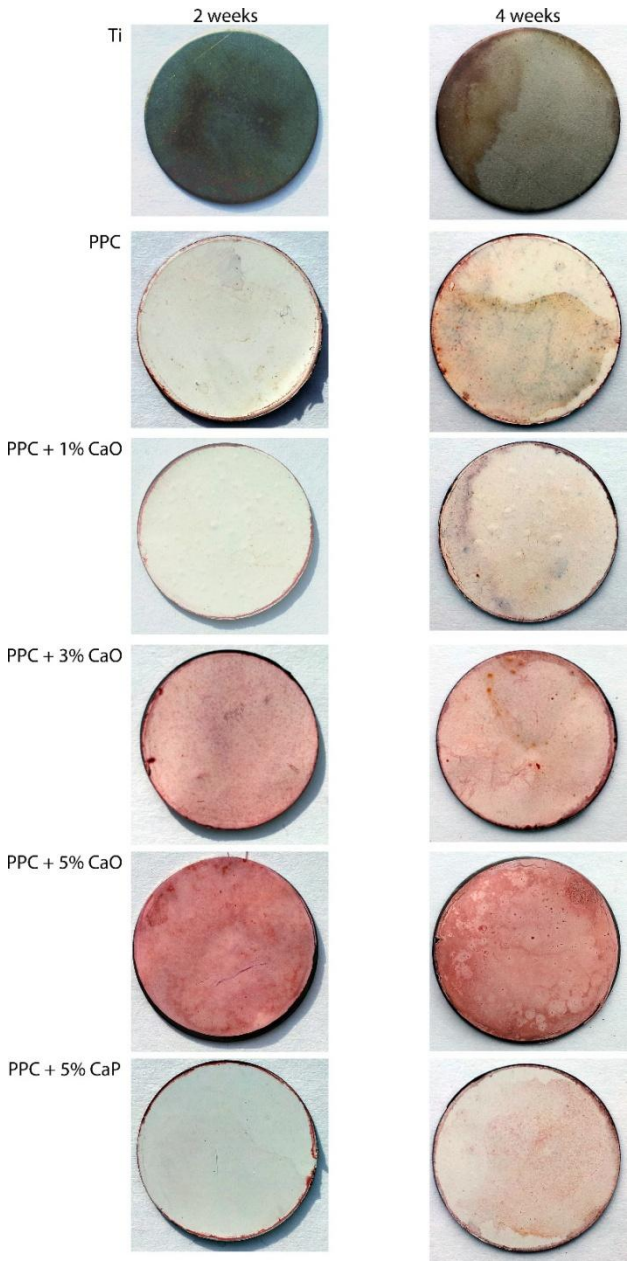

Figure 8. Biomineralized cell cultures were stained with Alizarin Red-S. After 2 weeks of growth and differentiation, there were numerous mineral deposits on the PPC + 3% CaO and PPC + 5% CaO coatings, and after 4 weeks there were also a few deposits on the other surfaces.

4. Discussion

This study has confirmed our prior research, which showed that a novel electrostatic ultrafine dry powder coating technique [19,20] can be used to create biomaterial surfaces [21–23]. This dry powder

coating technique has an advantage over conventional surface coating processes in that it eliminates the need to use toxic solvents and volatile organic compounds in the coating process, and thereby from the final product. Additionally, it can create highly biocompatible coatings that contain nano-scale surface topographies and roughness, which induce favorable cellular responses.

Furthermore, this research has confirmed that the surface coatings can be readily enriched with bioactive agents, which then serve as functional additives. This was initially done with ProRoot® MTA powders [18], which are a ceramic-like mixture of mineral oxides that are widely used in dentistry [24,34]. However, we have now shown that either CaO or $Ca_3(PO_4)_2$ alone can be used [25]. These are added alongside other additives in the process of preparing ultrafine powders, and account for only a nominal modification to the base formulation (PPC). They were limited to a maximum of 5% by weight, to ensure the continuity and homogeneity of the base coating. Whereas the original preparations were restricted by the amorphous composition of MTA mixtures, the refined formulations with CaO and $Ca_3(PO_4)_2$ can deliver calcium and phosphorous at higher levels.

Remarkably, these small enrichments were readily detected within the coating surfaces. There were small amounts of calcium in the CaO-enriched coatings, and both calcium and phosphorous in the $Ca_3(PO_4)_2$ coatings, when analyzed by EDX. Furthermore, their calcium content reflected their formulation, so that it increased progressively from the PPC + 1% CaO, to the PPC + 3% CaO and the PPC + 5% CaO surfaces. For CaO, there is one calcium for every oxygen, so that ample calcium was detected when even minimal amounts (1% w/w) were used. However, for $Ca_3(PO_4)_2$ there are three calcium, two phosphorus and eight oxygen that account for a much higher molecular weight. Therefore, these additives delivered less calcium or phosphorus when even high concentrations (5% w/w) were used. Yet, despite these modifications, overall chemical compositions were largely unchanged, and were very similar in all of the coatings [25].

In addition to the physiochemical characterization of these novel calcium-enriched coatings, it was necessary to evaluate the cellular response to their surfaces and confirm biocompatibility. In this study we found that all of the coatings supported human mesenchymal cell attachment and growth, as they did on the commercially pure titanium (cpTi) surfaces. The cells attached and spread out onto coatings and control surfaces within 24 hours of seeding, as demonstrated by SEM. They attached firmly and spread out with cellular filopodia that suggest intimate surface interactions. Dense cell cultures were observed on all of the surfaces within 24 hours of seeding, and they increased markedly over 72 hours of growth and proliferation. Additionally, the coatings sustained ongoing cell metabolism on their surfaces, which was measured as mitochondrial enzyme activity in the MTT assays. Indeed, prior studies found that similar surface coatings were able to support human mesenchymal cell attachment, metabolic activity and proliferation [21–23], and that their enrichment with ceramic-like additives, did not reduce their biocompatible features [18,25].

These favorable cellular responses were amplified by enriching the surface coatings with bioactive agents. Cell counts were higher on all CaO-enriched coatings than on the titanium controls, within 24 hours of seeding. Furthermore, their counts increased progressively with the increase in CaO content in the formulations (1, 3, 5% w/w). Although some of these increases were not statistically significant, a distinct trend was evident, and the PPC + 5% CaO coatings had the most cells on their surfaces. PPC + 5% CaO coatings had significantly (P = 0.006) more cells on their surfaces within 24 hours of seeding, than any of the other enriched or unenriched coatings or titanium controls. Yet, after 72 hours of growth and proliferation, cell counts became similar on all surfaces. Therefore, it appears that the small amounts of calcium in the surface coatings had actually promoted human mesenchymal cell attachment and spreading, without altering their subsequent rates of proliferation.

This increase in cell attachment and spreading on CaO-enriched coatings was accompanied by an increase in metabolic activity and downstream events. Cultures grown on CaO-enriched coatings had higher metabolic activities than those on the unenriched PPC coatings and titanium controls. Furthermore, the cultures grown on PPC + 5% CaO surfaces had the very highest levels of activity. Subsequently, the PPC + 3% CaO and the PPC + 5% CaO coatings were the first to show initiation of mineralization within 2 weeks, and widespread mineral deposits after 4 weeks of extended culture. This biomineralization was detected by Alizarin Red-S staining, which binds specifically to mineral deposits through its sulfonic acid and hydroxyl groups [35]. These experiments showed that CaO-enriched coatings had induced osteogenic differentiation of human mesenchymal cells into a pre-osteoblast-like phenotype, which could initiate bone-like mineral formation. Similarly, a prior study showed that the coating enrichment appeared to promote mineralization, and that the PPC + 5% CaO surfaces had a plethora of mineral deposits after 4 weeks [25]. Another study found that silica-calcium phosphate coatings increased alkaline phosphatase activity in bone marrow mesenchymal stem cells, which indicated an increase in osteogenic differentiation [30].

These effects may have been mediated by the surface calcium alone. Since a recent study found that merely the pre-treatment of implant surfaces with calcium solutions increased bone formation [26]. They found increased bone volume and density within 2 weeks, and bone contact after 8 weeks of implantation in rabbit femora. They found that calcium-treated implant surfaces promoted clot formation, platelet adsorption and activation when exposed to blood plasma. Therefore, the enhanced human mesenchymal cell responses that were discovered in our in vitro studies may become amplified in an in vivo setting, with the added presence of plasma, platelets and clot formation.

In contrast to this enhanced response to the CaO-enriched surfaces, the response to $Ca_3(PO_4)_2$-enriched coatings was largely subdued. The human mesenchymal cell attachment, spreading, proliferation, metabolic activity and biomineralization on PPC + 5% $Ca_3(PO_4)_2$ coatings were comparable to those on unenriched PPC surfaces and titanium controls. These coatings were clearly biocompatible, but did not possess the inductive effects of the CaO-enriched surfaces, particularly the PPC + 5% CaO. This may

have been due to low calcium and phosphate incorporation within their surfaces, given the high oxygen content of high molecular weight $Ca_3(PO_4)_2$ additives. It may also have been due to the greater importance of calcium over phosphate for protein binding [36] and cell attachment [37], and an inhibitory effect of $Ca_3(PO_4)_2$ on cell spreading [38]. Yet, several other studies have reported enhanced effects from $Ca_3(PO_4)_2$ modifications. They include increased bone formation in rat femora [27], enhanced healing in dog mandibles [28], and better retention in rabbit tibia [29], when $Ca_3(PO_4)_2$-coated titanium implants were used. Thus, additional animal studies will need to be performed to determine if the enhanced performance of CaO-enriched surfaces are sustained in vivo.

In conclusion, this study reported on an innovative surface modification for titanium implants that clearly warrants further investigation. It has shown that surface enrichments with CaO additives alone can markedly enhance the human mesenchymal cell response to biocompatible surfaces. These surface coatings can be prepared by a novel ultrafine dry powder coating technique that uses polyester resins and TiO_2 powders to create a polymeric base, which can be readily enriched with functional additives. The bioactive additives include ceramic-like mixtures of mineral oxide powders, and $Ca_3(PO_4)_2$ or CaO alone. Whereas most prior studies reported on the beneficial response to hydroxyapatite-like $Ca_3(PO_4)_2$ surfaces, this study documented a more heightened response from CaO additives alone.

Conflict of Interest

All authors declare no conflicts of interest in this paper.

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