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INFLUENCE OF SURFACE MICROSTRUCTURE AND CHEMISTRY ON OSTEOSTEOINDUCTION AND OSTEOCLASTOGENESIS BY BIPHASIC CALCIUM PHOSPHATE DISCS

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Abstract

It has been reported that surface microstructural dimensions can influence the osteoinductivity of calcium phosphates (CaPs), and osteoclasts may play a role in this process. We hypothesised that surface structural dimensions of ≤1 μm trigger osteoinduction and osteoclast formation irrespective of macrostructure (e.g., concavities, interconnected macro pores, interparticle space) or surface chemistry. To test this, planar discs made of biphasic calcium phosphate (BCP: 80% hydroxyapatite, 20% tricalcium phosphate) were prepared with different surface structural dimensions – either ~1 μm (BCP1150) or ~2-4 μm (BCP1300) – and no macro pores or concavities. A third material was made by sputter coating BCP1150 with titanium (BCP1150Ti), thereby changing its surface chemistry but preserving its surface structure and chemical reactivity. After intramuscular implantation in 5 dogs for 12 weeks, BCP1150 formed ectopic bone in 4 out of 5 samples, BCP1150Ti formed ectopic bone in 3 out of 5 samples, and BCP1300 formed no ectopic bone in any of the 5 samples. 

Introduction

Bone formation in dogs

It has been reported that surface microstructural dimensions can influence the osteoinductivity of calcium phosphates (CaPs), and osteoclasts may play a role in this process. We hypothesised that surface structural dimensions of ≤1 μm trigger osteoinduction and osteoclast formation irrespective of macrostructure (e.g., concavities, interconnected macro pores, interparticle space) or surface chemistry. To test this, planar discs made of biphasic calcium phosphate (BCP: 80% hydroxyapatite, 20% tricalcium phosphate) were prepared with different surface structural dimensions – either ~1 μm (BCP1150) or ~2-4 μm (BCP1300) – and no macro pores or concavities. A third material was made by sputter coating BCP1150 with titanium (BCP1150Ti), thereby changing its surface chemistry but preserving its surface structure and chemical reactivity. After intramuscular implantation in 5 dogs for 12 weeks, BCP1150 formed ectopic bone in 4 out of 5 samples, BCP1150Ti formed ectopic bone in 3 out of 5 samples, and BCP1300 formed no ectopic bone in any of the 5 samples. 

In vivo, large multinucleated osteoclast-like cells densely colonised BCP1150, smaller osteoclast-like cells formed on BCP1150Ti, and osteoclast-like cells scarcely formed on BCP1300. In vitro, RAW264.7 cells cultured on the surface of BCP1150 and BCP1150Ti in the presence of osteoclast differentiation factor RANKL (receptor activator for NF-κB ligand) proliferated then differentiated into multinucleated osteoclast-like cells with positive tartrate resistant acid phosphatase (TRAP) activity. However, cell proliferation, fusion, and TRAP activity were all significantly inhibited on BCP1300. These results indicate that the material parameters tested – namely, surface microstructure, macrostructure, and surface chemistry – microstructural dimensions are critical in promoting osteoclastogenesis and triggering ectopic bone formation.

Keywords: Biphasic calcium phosphate, topography, microstructure, osteoclast, osteoinduction.

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Certain calcium phosphates (CaPs) can induce de novo bone formation without exogenous stem cells or growth factors, making them particularly attractive for use as bone graft substitutes (Ripamonti, 1991; Yuan et al., 2010). Although the material parameters and biological signalling necessary to induce de novo bone formation are unclear, osteoinductive CaPs developed by different groups seem to share similar surface structure, specifically surface topographical features on a (sub)micron-scale. For instance, hydroxyapatite (HA) with surface micrograins and micropores induced ectopic bone formation in dogs and goats, but HA with a denser surface of large, fused grains and few micropores did not (Habibovic et al., 2005b; Yamasaki and Sakai, 1992; Yuan et al., 1998; Yuan et al., 1999). Similarly, microstructured biphasic calcium phosphate (BCP) – a mixture of HA and tricalcium phosphate (TCP) – induced de novo bone formation in the muscle of sheep (Le Nihouannen et al., 2005), goats (Habibovic et al., 2005b; Yuan et al., 2002), and dogs (Yuan et al., 2010); however, BCP with larger grains and fewer micropores induced less bone formation (Yuan et al., 2010) or in other cases none at all (Habibovic et al., 2006b).

More recently, the dimensions of surface microstructure have also been shown to be important for osteoinduction – for instance, TCP with submicron-scale surface structure consistently stimulated de novo bone formation in dog muscle, while TCP with micron-scale surface structure was not at all osteoinductive (Davison et al., 2014b; Zhang et al., 2014). Surface microstructure may also be critical in triggering osteoinduction by other biomaterials such as titanium (Fujibayashi et al., 2004; Fukuda et al., 2011).

Macroscale features of osteoinductive biomaterials such as interconnected macro pores, particle size, and surface concavities have also been previously speculated to be “essential” and “requisite” for de novo bone formation (Habibovic et al., 2005a; Habibovic et al., 2005b; Magan and Ripamonti, 1996; Yuan et al., 2002). However, extensive de novo bone can also form in the intramuscular space between non-macroporous, microporous CaP particles (Yuan and de Bruijn, 2011). Thus, it is still unclear if interparticle space along with microstructure is necessary for osteoinduction or if de novo bone can also form on a macroscopically flat surface.

The physicochemical properties of CaPs are also theorised to be crucial for osteoinduction through the formation of a crystalline carbonate apatite surface layer after implantation (Daculsi et al., 1989; LeGeros, 2008).
The solubility of a given CaP (e.g., the HA/TCP ratio in the case of BCP), as well as its microstructure (e.g., surface micropore and crystal grain size) contribute to this mineralised surface layer by modulating the dissolution/reprecipitation of calcium and phosphate ions in body fluid (Daculsi et al., 1990). The biological relevance of surface reactivity and a precipitated layer of carbonate apatite is speculated to be either a direct physicochemical trigger for osteogenesis (i.e., the differentiation of bone forming osteoblasts from uncommitted precursors) through elevated local calcium and phosphate levels (Barradas et al., 2013; Beck et al., 2000; Syed-Picard et al., 2013), or a biomimetic template for bone deposition following osteoblast differentiation by another means (LeGeros, 2008). However, surface reactivity and carbonate apatite precipitation may only be a permissive factor in osteoinduction, not an osteogenic trigger. Taking the case of osteoinductive titanium as an example, Fujibayashi et al. (2004) reported that although both titanium mesh cylinders and porous blocks formed an apatite layer in simulated body fluid in vitro after thermochemical treatment, only the porous blocks induced ectopic bone formation – potentially due to more complex surface topography (Fujibayashi et al., 2004). Because surface reactivity and physical topography can both influence osteoblast differentiation but are linked to surface architecture, it is currently unknown which, if either, material property plays a prevailing role in osteoinduction (Curran et al., 2006; Habibovic et al., 2006a; Vlacic-Zischke et al., 2011; Zhao et al., 2007).

Alternatively, osteoinduction may depend on (pre-) osteoclast activity for osteogenic signals rather than intrinsic physicochemical signals originating from the material itself (Baslé et al., 1993; Gauthier et al., 2005; Malard et al., 1999). In support of this theory, it has been reported that osteoindigestion precedes osteoinduction by microstructured TCP by several weeks (Akiyama et al., 2011; Kondo et al., 2006), and osteoclast depletion limits (Ripamonti et al., 2010) or completely blocks de novo bone formation by osteoinductive CaPs (Davison et al., 2014a). Recently, we reported a clear link between TCP microstructure, osteoindigestion, and subsequent de novo bone formation (Davison et al., 2014a; Davison et al., 2014b). However, (pre-)osteoclast differentiation and activity is influenced by multiple substrate parameters including surface nano-/microroughness (Makihira et al., 2007; Webster et al., 2001), solubility (Benahmed et al., 1996; Yamada et al., 1997), and the accompanied release of nano-/microparticulate (Fellah et al., 2007; Velard et al., 2013), so it is currently unknown if this link also holds true for less resorbable materials like BCP or titanium.

Given the present knowledge, we hypothesised that surface structure is the preeminent material factor responsible for the formation of both osteoclast-like cells and de novo bone. To evaluate this, two BCPs with different surface structure were prepared in the form of planar, non-macroporous discs, thus eliminating the effects of interconnected macropores, concavities, or interparticle space. To evaluate whether the surface chemistry contributes to osteoinductivity, BCP was also surface coated with titanium. Disc constructs were implanted in the dorsal muscle of dogs, the classical model for evaluating osteoinductivity, and the formation of de novo bone and multinucleated osteoclast-like cells was analysed by histology. The effects of surface structure and chemistry on osteoclastogenesis were further evaluated in vitro using the RAW264.7 pre-osteoclast cell line, as previously described (Davison et al., 2014b). Osteoclast differentiation, survival and morphology were measured and quantitatively compared using several biochemical, histological and morphological techniques.

Materials and Methods

Preparation and characterisation of BCP

BCP powder composed of 80% HA/20% β-TCP was prepared by wet precipitation as described elsewhere (Yuan et al., 2002). The powder was foamed with diluted H₂O₂ (0.1%) (Merck, Schipholt-Rijk, Netherlands) at 60 °C to produce microporous green bodies and then dried. The dry green bodies were subsequently sintered at 1150 °C or 1300 °C for 8 h to achieve surface micro-grains and pores (BCP1150) or larger fused grains and few micropores (BCP1300). Ceramic discs (Ø 9 x 1 mm) were machined from the ceramic bodies using a lathe and a diamond saw microtome (Leica SP1600). Discs were ultrasonically cleaned in successive baths of acetone, ethanol and deionised water for 15 min, and then dried at 60 °C.

To obtain a different surface chemistry while preserving the surface microstructure, BCP1150 discs were sputter coated with titanium (BCP1150Ti) using a radiofrequency magnetron unit (Edwards ESM 100) as previously described (Wolke et al., 1998). Both sides of the discs were coated for 15 min at 200 W, resulting in a visually complete layer of titanium roughly 50 nm thick. The elemental composition and distribution of the titanium coating was verified using electron dispersive spectroscopy (EDS), as previously described (Bongio et al., 2013). Briefly, samples were affixed to metal stubs and scanned by a scanning electron microscope (Philips XL30) equipped with an energy dispersive spectrometer (EDAX, Ametek). The distribution of elements of interest (Ca, P and Ti) was analysed and visually displayed. The associated error for all the EDS analyses was calculated to be less than 10%.

Surface structure of the materials was characterised by scanning electron microscopy (SEM) (JEOL JSM-5600) after sputter coating with gold for 90 s (JEOL JFC 1300). Surface grain and pore size were quantified in scanning electron micrographs (magnification: 5000×, n = 3 random locations) by measuring the vertical distance across the features (n > 50) in Image J software (NIH, Bethesda, MD, USA).

Crystal chemistry of the materials was analysed by X-ray diffraction (Rigaku Miniflex II) scanning the range 2θ = 20–45° (step size = 0.01°, rate = 1° min⁻¹) as previously described (Davison et al., 2014b). The surface reactivity of the discs was analysed in simulated physiologic solution (SPS) (50 mM HEPES, 140 mM NaCl, and 0.4 mM Na₂ for sterility; all from Sigma Aldrich, Saint Louis, MO, USA) at pH 3 and pH 7. Discs (n = 3) were incubated in
SPS (8 mL) in a tissue culture multiwell plate incubated at 37 °C, 5 % CO₂ for 7 d with gentle shaking. The solution was sampled and refreshed with the same amount (100 μL) after 10 min, 1, 2, 4 h, 1, 4 and 7 d. Calcium and phosphate released into the solution were quantified using QuantiChrom (BioAssay System, Hayward, CA, USA) and PhoshoWorks (AAT BioQuest, Sunnyvale, CA, USA) colorimetric assay kits, respectively, following the manufacturers’ instructions. Absorbance was detected using a Zenyth 3100 multimode spectrophotometer.

In vivo study of osteoinduction by BCP constructs

Implantation of sandwich constructs

BCP constructs were implanted in the dorsal muscle of dogs to test their capacity to form ectopic bone. BCP constructs were made by gluing (Cyanoacrylate “Superglue”, Pertex, Cornwall, UK) two discs together with two strips of nylon wire (~0.7 mm) in between to create a central gap (Fig. 3A). “Sandwich” shaped constructs were sterilised by gamma irradiation (>25 kGy) prior to implantation.

All surgery was conducted at the Animal Centre of Sichuan University in conformance with the institutional animal ethics committee’s guidelines. Sterile BCP constructs were implanted in the dorsal muscle of healthy male mongrel dogs (n = 5 dogs, 1-4 years, 10-15 kg) for 12 weeks. Animals were first given general anaesthesia by abdominal injection of sodium pentobarbital (30 mg kg⁻¹ body weight) and constructs were implanted into paraspinal muscle pockets created by scalpel incision and blunt dissection. One construct of each material was implanted in each dog resulting in 3 constructs implanted per animal. Skin incisions were closed layer by layer with non-resorbable sutures for identification at harvest. Following surgery, the animals were given daily intramuscular injections of buprenorphine (0.1 mg per animal) for 2 d and penicillin (40 mg kg⁻¹) for 3 d to relieve pain and prevent infection. Animals were allowed to undertake full activity and received a normal diet immediately after surgery.

Sample harvest and histological processing

At the end of 12 weeks, the animals were euthanised by abdominal injection of sodium pentobarbital (60 mg kg⁻¹) and samples were immediately harvested and fixed in cold phosphate-buffered formalin solution, dehydrated in graded ethanol series, and embedded in methyl methacrylate (MMA) (LTI, Bilthoven, Netherlands) at room temperature. Histological sections (~30 μm) of the undecalcified samples were made using a Leica SP1600 microtome and stained en bloc with 1 % methylene blue and 0.3 % basic fuchsin solutions for histological analysis.

Stained histological sections were scanned using a Dimage Scan Elite 5400II slide scanner (Konica Minolta) for gross evaluation. Bone formation was analysed at 20× magnification using a light microscope (Nikon Eclipse E200). More than 10 sections per sample spanning more than half the construct were analysed for de novo bone formation by 2 investigators (ND and JS), and the number of samples positive for bone formation per the total number of samples implanted (i.e., bone incidence rate) was recorded.

In vitro studies

Culture of RAW264.7 osteoclasts and C2C12 myoblasts on BCP discs

To model osteoclastogenesis in vitro, murine RAW264.7 macrophages (ECACC, Salisbury, UK) were cultured on the surface of BCP discs for up to 5 d in the presence of osteoclast differentiation factor RANKL (receptor activator for NF-kB ligand) as described previously (Collin-Osdoby et al., 2003). RAW264.7 cells were first expanded in tissue culture flasks with basic medium composed of alpha MEM (Lonza, Breda, Netherlands), supplemented with 10 % HyClone FetalClone I serum (Thermo Scientific, Waltham, MA, USA) and 1 % penicillin-streptomycin (Life Technologies, Merelbeke, Belgium). At ~75 % confluence, cells were scraped loose from the tissue culture flasks, resuspended in basic medium supplemented with RANKL (40 ng mL⁻¹, Peprotech, London, UK), and seeded on BCP discs (2 × 10⁶ cells cm⁻²). All discs were heat sterilised in a dry chamber at 200 °C for 2 h prior to cell culture.

RAW264.7 cells were cultured for 5 d with medium refreshment (basic medium + RANKL) after 1 d. In our previous experience with this culture model (Davison et al., 2014b), cells begin to fuse and differentiate into osteoclasts by day 3, continue fusing through day 4-5, and undergo apoptosis by day 6-7 (Collin-Osdoby et al., 2003; Takahashi et al., 2007). Therefore, biochemical assays focused on day 3-5 as the relevant period of osteoclastogenesis. Osteoclast culture experiments were repeated to confirm the results of the various assays described below.

C2C12 myoblasts were cultured on BCP discs to study the effects material properties on muscle cells. C2C12 cells were similarly expanded in basic medium, trypsinised at confluence, and cultured on BCP discs (seeding density = 2 × 10⁴ cells cm⁻²) for 5 d. All cells were cultured in a humidified incubator maintained at 37 °C and 5 % CO₂.

Cell viability, proliferation, and DNA content

The AlamarBlue (AB) fluorescent assay (Life Technologies) was used to measure cell viability and proliferation (Nakayama et al., 1997) on BCP. AB measures the reductive activity inside living cells, and is commonly used in the literature as a more sensitive alternative to formazan-based cell viability assays such as MTT and XTT (Ahmed et al., 1994; Gloeckner et al., 2001). At various culture time points, cells were incubated with culture medium containing 5 % AB reagent for 2 h in culture conditions and then media samples were collected in a 96-well plate for fluorescent detection (excitation = 530 nm, emission = 590 nm) using a Zenyth Multimode plate reader. Cell proliferation can be measured by assaying cell viability over time (Nakayama et al., 1997). For this assay, the same procedure was followed except that AB-containing culture medium was removed and refreshed with normal culture medium, and then continuously cultured until the next time point. For viability and proliferation assays, n = 3 culture replicates were measured.

DNA content was measured in the cell lysate using a CyQuant DNA detection kit (Life Technologies). After 3, 4 and 5 d culture on discs, adherent cells were rinsed in phosphate-buffered saline (PBS) and then freeze-thawed.
in CyQuant cell lysis buffer, as recommended by the manufacturer. Cell lysate was thoroughly homogenised and sampled from \( n = 3 \) replicate discs for measurement using the kit. A Zenyth 3100 Multimode plate reader was used to detect the fluorescent signal of the assay.

**Tartrate resistant acid phosphatase (TRAP) activity**

Tartrate resistant acid phosphatase (TRAP) activity, an enzyme marker of osteoclast differentiation (Halleen et al., 2001), was measured in RAW264.7 cells cultured on discs after 3, 4 and 5 d by both biochemical activity and cytochemical staining. TRAP activity in the cell lysate from \( n = 3 \) culture replicates was quantified by conversion of \( p \)-nitrophenylphosphate to \( p \)-nitrophenol (pNP) in sodium acetate buffer (pH 5.8) containing potassium sodium tartrate (10 mM), as reported by Ljusberg et al. (1999). Cell lysate was obtained by first rinsing disc-adherent cells with PBS and then freeze-thawing in cell lysis buffer (0.1 M sodium acetate, 0.1 % Triton X-100, pH 5.8). All reagents were purchased from Sigma Aldrich. Optical absorbance of the assay reaction was measured using a Zenyth multimode spectrophotometer. Absorbance was converted to mM pNP using a standard curve of pNP (Sigma Aldrich) and normalised to viable cell signal from AlamarBlue. TRAP was also visualised on \( n = 2 \) disc replicates using a commercial staining kit (Leukocyte Acid Phosphatase Kit, Sigma Aldrich). Prior to staining, cells were briefly rinsed in PBS and fixed in acetone methanol solution as per the manufacturer’s instructions. Images were captured using a Nikon SMZ800 stereomicroscope equipped with a Nikon camera.

**SEM of osteoclast morphology**

Osteoclast morphology was analysed by SEM. Cells cultured on discs (\( n = 2 \)) were fixed in 2.5 % glutaraldehyde, dehydrated in a graded ethanol series, and finally dried in hexamethyldisilazane (HMDS; Alfa Aesar, Karlsruhe, Germany). Dehydrated cells were then sputter coated with gold for enhanced imaging resolution. Osteoclast size was quantified in scanning electron micrographs (400× magnification), by calculating the mean surface area of cells at 3 random locations of replicate discs (\( n = 2 \)) using automated threshold, edge detection, and particle analysis functions in ImageJ software (NIH), as previously described (Davison et al., 2014b). Only cells > 400 \( \mu \)m\(^2\) were included in the analysis to safely exclude mononuclear cells.

**Statistical analysis**

Statistical comparisons were performed using One-way ANOVA and Tukey’s post hoc tests; \( p \) values < 0.05 were considered significant. All statistical analyses were conducted in GraphPad Prism 6.0.

**Results**

**BCP characterisation**

BCP1150 and BCP1300 with different surface microstructures were prepared by changing the sintering temperatures, as shown by SEM (Fig. 1A). Quantitatively, BCP1150 contained grains and pores sized \( \leq 1 \mu \)m in diameter but BCP1300 contained larger, fused grains...
(~ 3 μm) and larger but fewer micropores (~ 2 μm) (Fig. 1B)). Because no macropore porogens were introduced during synthesis, neither material contained macropores or substantial concavities. Sputter coating BCP1150 with titanium (BCP1150Ti) did not visibly change the surface microstructure by SEM (Fig. 1A) or the size of the surface grains and pores (Fig. 1B) versus BCP1150.

The crystal chemistry of the materials was confirmed by X-ray diffraction (XRD) to be BCP containing 80-85 % HA and 15-20 % TCP (Fig. 2A). Coating BCP1150 with titanium did not substantially alter the XRD spectra. The surface reactivity of the materials was analysed by measuring calcium and phosphate ion release in simulated physiologic solution (SPS) at pH 7 and pH 3 (Fig. 2B). At neutral pH, all three materials released similar amounts of ions over time, but at acidic pH, ion release from BCP1150 and BCP1150Ti was higher than BCP1300, resulting from the increased surface area of the microstructure. There was no change in ion release by BCP1150 with or without the titanium coating showing that the coating did not change the chemical reactivity of the material (Fig. 2B).

Sputter coating BCP1150 with titanium resulted in a visually homogenous layer on all sides of the discs (Fig. 3A). The titanium layer was analysed by EDS, which showed the homogeneously distributed titanium coating on the surface (Fig. 3B) that remained on the surface after implantation (Fig. 4). In summary, sputter coating BCP1150 with titanium resulted in a material with equivalent microstructure and chemical reactivity but different surface chemistry.

In vivo results
BCP sandwich constructs were implanted into the dorsal muscle of dogs for 12 weeks to study the effects of surface microstructure and chemistry on osteoinduction. A gap between the BCP discs was created using nylon wire spacers to allow tissue in-growth and bone formation (Fig. 5A). However, soft tissue formation in the space between the discs and around the nylon wires tended to be weak for all materials compared to tissue formation on the outer edges of the constructs (Fig. 5B).

The incidence of de novo bone formation was quantified by thorough analysis of histological sections. De novo bone formation was observed in 4 out of 5 BCP1150 constructs, 3 out of 5 BCP1150Ti constructs, and 0 out of 5 BCP1300 constructs (Table 1). For BCP1150 and BCP1150Ti, bone was predominantly formed on the outer surfaces of the constructs (Fig. 6A) rather than on the inner surfaces of the central gap. Although stretches of bone were not thicker

![Fig. 2. Chemical characterisation of BCP. The XRD spectra were equivalent for all three BCP materials (A). Chemical reactivity in simulated physiologic solution (SPS) showed that ion release of all three materials was equivalent at pH 7, but slightly faster for BCP1150 with and without titanium coating than BCP1300 at pH 3 (B). Data represents the mean ± S.D. of n = 3 replicate discs, p < 0.0001.](image-url)
Fig. 3. Elemental analysis of BCP1150Ti by electron dispersive spectroscopy (EDS). (A) Overview images of BCP1150 and BCP1150Ti show that discs were appreciably devoid of concavities or macropores and that titanium coating uniformly covered the disc surfaces. (B) Elemental diffraction spectroscopy (EDS) analysis (2,000× magnification) shows titanium (Ti) on the surface of BCP1150Ti (left column) was evenly distributed and porous similar to the underlying substratum composed of calcium (Ca) and phosphorus (P). In comparison, BCP1150 (right column) depicted only background noise. Scale = 10 μm.
**Fig. 4.** Elemental analysis of BCP1150Ti explant. After 12 weeks intramuscular implantation, a thin layer of titanium was still intact on the edge of the BCP115Ti construct cross-section. Scale = 50 μm.

**Fig. 5.** Intramuscular implantation of BCP sandwich constructs. BCP sandwich constructs were made by gluing together two BCP discs with a central gap in between them using nylon wire spacers (A). Constructs were implanted in the dorsal muscle of dogs for 12 weeks and histological sections were stained with methylene blue and basic fuchsin (B). Overview images of cross-sections taken through the middle of explants show soft tissue (pink, purple, blue) formation around the BCP constructs (brown, black) with limited tissue infiltration in the gap between the discs. Tissue often delaminated from the surface of BCP1300 constructs (black arrows), indicating weak tissue bonding. Note: few macropores or concavities were present in the discs. B, scale = 1 mm.
than ~ 50 μm and generally spanned less than several hundred μm long, cuboidal osteoblasts were seen forming new bone and osteocytes were present in bone lacunae (Fig. 6A). Bone area was not quantified by histomorphometry due to the small amounts present.

Multinucleated osteoclast-like cells extensively covered the surface of BCP1150, but were smaller and less organised on BCP1150Ti (Fig. 6B). For both materials, osteoclast-like cells adhered to the material adjacent to de novo formed bone. In contrast to BCP1150, with and without a titanium coating, BCP1300 was largely encapsulated by fibrous tissue and contained scarce multinucleated osteoclast-like cells (Fig. 6B).

**In vitro results**

**Cell viability and proliferation**

To further investigate the effects of BCP surface structure and chemistry on osteoclast-like cell formation, RAW264.7 macrophages were cultured on BCP discs and differentiated into osteoclast-like cells using RANKL. At day 3, 4 and 5,
DNA content from cells cultured on BCP1150 was ~3-5 times greater than on BCP1300 (day 3: \( p < 0.0001 \); day 4: \( p = 0.0001 \); day 5: \( p = 0.004 \)) (Fig. 7A). DNA content from cells cultured on BCP1150Ti was also significantly greater than on BCP1300 (\( p < 0.01 \)), at levels similar to BCP1150 at day 4 and 5 (Fig. 7A). These data indicated that the difference in titanium coating had little effect on cell growth; however, the difference in microstructure had a pronounced effect. After 5 d of culture, cell viability was ~2× higher on both BCP1150 and BCP1150Ti than on BCP1300 (both \( p < 0.0001 \)) (Fig. 7B). Further, cell viability was higher for BCP1150Ti than for BCP1150 (\( p = 0.002 \)) (Fig. 7B).

RAW264.7 cell proliferation was analysed on BCP1150 and BCP1300 by measuring cell viability over time normalised to the viability at the time of seeding (d0) (Fig. 7C). BCP1150Ti was not included in this analysis, focusing only on the effects of surface structure, not surface chemistry. At day 1, cell viability was similar on the materials suggesting that initial cell attachment was equivalent. By day 3, RAW264.7 cell proliferation was significantly greater for BCP1150, resulting in ~2× greater viability than on BCP1300 (\( p = 0.001 \)). The same difference in cell viability was maintained through day 4 (\( p = 0.004 \)) and day 5 (\( p = 0.005 \), indicating that BCP1150 stimulated significantly more proliferation of RAW264.7 cells than BCP1300 over the entire culture period. In fact, RAW264.7 cells cultured on BCP1300 did not proliferate between 1 and 5 d in culture (Fig. 7C). To evaluate if interactions with BCP1300 inhibited the proliferation of other cell types, C2C12 myoblasts were also cultured on the materials, but in contrast, these cells proliferated in typical logarithmic fashion on BCP1300 and to a greater extent than on BCP1150 by day 4 and 5 (\( p = 0.003 \) and \( p = 0.001 \), respectively) (Fig. 7D).

In sum, BCP1150 promoted significantly higher cell growth and viability of RAW264.7 (pre-)osteoclasts than BCP1300 in a process that was not adversely affected by...
titanium coating; however, this response was not universal to other cell types such as C2C12 myoblasts.

**TRAP activity**

TRAP enzyme activity in the RAW264.7 cells was assayed both biochemically in the cell lysate and cytochemically by staining. Biochemical TRAP activity in the lysate of cells cultured on BCP1150 was significantly higher than that of BCP1300 at day 3 (~4×, \( p < 0.0001 \)), day 4 (~3×, \( p < 0.0001 \)) and day 5 (~2×, \( p = 0.008 \)) (Fig. 8A). Cells cultured on BCP1150Ti also expressed significantly more TRAP activity than BCP1300 at day 3 (~2×, \( p = 0.023 \)) and day 4 (~2.5×, \( p = 0.002 \)), although at day 5 there was no statistical difference (\( p = 0.194 \)). Cellular TRAP activity was different between BCP1150 and BCP1150Ti at day 3 (~1.8×, \( p = 0.004 \)); however, by day 4 and 5 there was no statistical difference (\( p = 0.144 \) and 0.102, respectively) (Fig. 8A).

To visually confirm the biochemical results, cells were stained for TRAP at the same time points (Fig. 8B).
Visualisation of TRAP staining on BCP1150Ti was not possible because of the dark colour of the coating. A clear difference in osteoclast fusion and TRAP activity between BCP1150 and BCP1300 was observed (Fig. 8B): cells were substantially larger and more intensely stained on BCP1150 at all time points. On BCP1150, numerous cell-cell junctions were observed between densely distributed cells; in contrast, on BCP1300, cell junctions were sparse likely owing to less cells present (Fig. 8B), in confirmation of the cell viability and DNA assays (Fig. 7).

Osteoclast morphology and size
Osteoclast morphology and size were analysed by SEM at day 5, corresponding with the peak of TRAP activity and cell fusion visualised by TRAP staining (Fig. 9). On BCP1150, fused cells were massive (~ 4,000 μm²) and tightly attached to the BCP surface in an extensive cell network. Single cells were generally found in clusters, with partially fused cell membranes. In contrast, fused cells on BCP1150Ti were ~ 75% smaller (~ 1,000 μm², p = 0.002) and appeared rounder and less spread out on the surface. On BCP1300, fused cells were also smaller than on BCP1150 (~ 1,500 μm², p = 0.008), and often appeared to be apoptotic or necrotic with deteriorating cell membranes. Fewer cells were present on BCP1300 than BCP1150 and BCP1150Ti, in agreement with the cell viability and DNA assays (Fig. 7).

Discussion
In the present results, BCP and titanium-coated BCP with small surface microstructural dimensions (~ 1 μm) promoted osteoclast-like cell formation along with de novo bone formation, while larger surface architecture (~ 2-4 μm) inhibited these effects. Moreover, macro-scale features such as concavities, macropores, or interparticle space were unnecessary to stimulate this response. These in vivo observations were further investigated in vitro using a previously described osteoclastogenesis model (Davison et al., 2014b). Notably, osteoclast survival and differentiation were significantly promoted by the osteoinductive surface structure of BCP1150 and BCP1150Ti versus the non-inductive surface structure of BCP1300. Pre-osteoclast proliferation was also stunted by BCP1300 versus BCP1150; however, C2C12 myoblasts proliferated strongly on BCP1300 versus BCP1150,
illustrating that BCP1300 was not universally detrimental to cell proliferation. These in vitro results may also explain why few multinucleated cells but abundant soft tissue was present on this surface in vivo. Regarding osteoclast fusion and size, BCP1150 stimulated the formation of large, fused osteoclasts that were ~2-4 times larger than those formed on either BCP1150Ti or BCP1300 in vitro. In this way, surface microstructural dimensions of ~1 μm promoted (pre-)osteoclast proliferation, differentiation, and survival versus larger surface structure, while titanium surface chemistry appeared to limit osteoclast fusion.

At the onset of the present study, it was unclear whether planar, macroscopically flat implants could induce bone formation, based on a lack of direct investigation in the literature (Barradas et al., 2011). Bone formed on the outside surface of the microstructured constructs, not only between the discs; thus, the crucial role of surface microstructure on osteoinduction was more clearly isolated and interparticle space was shown to be dispensable. Still, the amount of ectopic bone formed in the present study was small in comparison to the ectopic bone formed by a similar microstructured BCP with macroporous structure, as previously reported (Habibovic et al., 2006b; Yuan et al., 2010). So, macrostructural features may enhance bone deposition after it has already been triggered by osteoinductive microstructure.

Ectopic bone also formed on the titanium surface of BCP1150Ti indicating that surface chemistry is a flexible parameter in the osteoinductive performance of microstructured materials. Rather than being fully sealed, the line-of-sight sputter deposition of titanium on BCP1150 preserved the chemical reactivity of the BCP substrate and was still intact after implantation. Whereas BCP1150Ti possessed small surface microarchitecture and similar dissolution profile of the underlying BCP1150 substrate, other osteoinductive titanium materials described in the literature possess nano-/microarchitecture (Fujibayashi et al., 2004; Fukuda et al., 2011), and being fully made up of titanium are incapable of releasing calcium or phosphate ions into solution. In a preliminary step toward the development of osteoinductive titanium, Kokubo (1996) showed that alkali followed by thermal treatment of pure titanium resulted in a stabilised microporous surface structure that could form a carbonate apatite layer in vitro and in vivo, and even bond directly to native bone (Kokubo, 1996; Kokubo et al., 1996). Tuning this alkali thermal treatment (10 M NaOH to 5 M NaOH) later resulted in a different nano-/microrough surface and the induction of de novo bone (Fujibayashi et al., 2004; Fukuda et al., 2011). However, in these same studies it was found that apatite formation alone was not sufficient to induce ectopic bone formation, despite the positive impact on osseointegration. Similarly, it is known that BCP readily forms a carbonate apatite layer in body fluid (Daculsi et al., 1989; Daculsi et al., 1990), which we also confirmed for BCP1150 and BCP1300 in simulated body fluid (data not shown); however, only BCP1150 – and now BCP1150Ti with equivalent microstructure – can induce ectopic bone formation. In agreement with the conclusion of Fujibayashi et al. (2004), we propose that these differences hinge on microarchitecture (i.e., topography) although apatite formation is likely a prerequisite for osteoinduction to take place because of its importance for bone-bonding. Considering that collagen fibres also infiltrate a microporous, osteoinductive surface before de novo bone formation (Kondo et al., 2006), apatite formation and microarchitecture may synergise to provide a biomimetic template for both phases of bone tissue.

Because BCP1150, BCP1150Ti and BCP1300 all shared similar Ca²⁺ and P, release profiles in vitro, the differences in bone formation are difficult to explain in terms of intrinsic differences in surface reactivity or Ca²⁺/P, signalling. However, this in vitro characterisation is limited in light of the physico-chemical complexity of body fluid in vivo, including supersaturated Ca²⁺/P, levels as well as blood serum (Garnett and Dieppe, 1990). Other theories on osteoinduction speculate that material degradation by osteoclast resorption or macrophage phagocytosis may independently speed the dissolution/precipitation of a bioactive carbonate apatite layer (LeGeros, 1993), establish an instructive geometric template for de novo bone formation in resorption lacunae along with increased local Ca²⁺ concentrations (Klar et al., 2013; Ripamonti et al., 2008; Wilkinson et al., 2011), or liberate crystalline nano-/microparticulate and a subsequent osteogenic cytokine cascade (Gauthier et al., 1999; Malard et al., 1999; Velard et al., 2013). However, in the present study neither characteristic osteoclast resorption lacunae nor degraded BCP particulate were apparent in the histology.

Alternatively, decades of research have shown that surface topography can directly stimulate bone cell differentiation and function on various material substrates, including polymers (Fu et al., 2010; Watari et al., 2012; Wilkinson et al., 2011; You et al., 2010), titanium (Brunette, 1988; Gittens et al., 2011; McNamara et al., 2011), ceramics (Webster, 2000; Zhang et al., 2014), and tissue (Gray et al., 1996). Topographical control of cell fate is a complex phenomenon that can occur through focal adhesion clustering and downstream focal adhesion kinase (FAK) signalling (McNamara et al., 2010). This cascade is initiated when cell surface integrins bind matrix proteins adsorbed to the substrate (Chou et al., 1995; Stevens and George, 2005), so protein adsorption from the body fluid may play a crucial role in the differences in cell-surface interactions observed in the present study. Indeed, our previous experiments showed that microstructured BCP1150 adsors more proteins than denser BCP1300 (Yuan et al., 2010).

With respect to the role of osteoclasts in osteoinduction, the present study further substantiates a link between microstructure, osteoclastogenesis, and eventual de novo bone formation. We previously reported similar findings using TCP with two different surface structures, analogous to BCP1150 and BCP1300 investigated in the present study (Davison et al., 2014b; Zhang et al., 2014). TCP possessing surface microstructural dimensions ≤1 μm (TCPs) was extensively colonised by multinucleated osteoclast-like cells adjacent to substantial amounts of ectopic bone in the muscle tissue of dogs after 12 weeks. In contrast, TCP with larger surface structural dimensions (~2-4 μm, TCPb) contained few multinucleated cells and formed no ectopic bone. Moreover, TCPs significantly promoted osteoclast
differentiation and fusion versus TCPb using the same in vitro osteoclastogenesis model applied in the present study (Davison et al., 2014b). Taking these previous and current results together, it can be concluded that for both BCP and TCP – representing the most frequently investigated osteoinductive materials in the literature (Barradas et al., 2011) – surface microstructural dimensions of ~1 μm robustly promoted the formation of osteoclast-like cells concurrent with de novo bone formation. These results add to the growing consensus that osteoclast formation is prerequisite for osteoinduction (Davison et al., 2014a; Klar et al., 2013; Kondo et al., 2006; Le Nihouannen et al., 2005); however, it is still unknown what the exact role of osteoclasts is in this process.

It has also been suggested that CaPs may stimulate bone formation by absorbing BMPs (bone morphogenetic proteins) endogenously synthesised near the implant surface (Klar et al., 2014; Ripamonti et al., 1993) or circulating in the blood (de Groot, 1998). However, large doses of BMPs are required to stimulate substantial amounts of de novo bone formation, likely rendering basal levels of BMPs circulating in the blood ineffective in achieving this response (van Baardewijk et al., 2013; Yuan et al., 2010). Alternatively, BMPs or other osteogenic factors may originate from (pre-)osteoclast interactions with microstructured surfaces including CaP (Davison et al., 2014b) and titanium (Takebe et al., 2003). Elevated Ca\textsuperscript{2+} levels resulting from osteoclast resorption of a mineralised substrate can also stimulate BMP expression of precursor cells (Barradas et al., 2012; Klar et al., 2013).

In support of this, osteoclast depletion by bisphosphonate treatment attenuated BMP2 expression in osteoinductive CaP implants and limited ectopic bone formation (Klar et al., 2013), potentially because osteoclasts synthesise a variety of BMPs (Garimella et al., 2008). Moreover, treatment with noggin, which blocks BMP binding to its membrane-bound receptor, also stunted ectopic bone formation by an osteoinductive CaP (Klar et al., 2014). However, chondrogenesis was not reported in either of these studies (Klar et al., 2013; Klar et al., 2014), or in a thorough review of osteoinductive materials research (Barradas et al., 2011), suggesting that osteoinduction may not proceed via a classical BMP-induced endochondral pathway. In the broader context of bone metabolism, activated (pre-)osteoclasts secrete a variety of other non-BMP osteogenic factors – e.g., Wnts, S1P, OSM, and CTHRC1 – resulting in osteoblast differentiation of local precursors (Garimella et al., 2008; Guihard et al., 2012; Pederson et al., 2008; Takeshita et al., 2013) through intramembranous ossification (Durmus et al., 2006). To elucidate the mechanism of osteoinduction, more research is needed to discern the distinct molecular pathways governing endochondral versus intramembranous ossification over the entire time course of ectopic bone formation.

To challenge the theory that osteoclast formation promoted by surface (sub)microstructure is instrumental for osteoinduction, osteoclastogenesis on other osteoinductive materials should be investigated. If, for example, microstructured HA and titanium also promoted osteoclastogenesis and ectopic bone in contrast to their non-microstructured controls, a broader link between osteoclast formation and de novo bone formation would be further substantiated. Pending deeper biological insight, it may be possible to anticipate osteoinductive performance based on simplified in vitro osteoclastogenesis models. And, if osteoclasts are not only requisite but also directive in de novo bone formation through the secretion of trophic factors, locally stimulating osteoclastogenesis (i.e., controlled release of RANKL) may even render non-microstructured CaPs osteoinductive.

**Conclusion**

BCP1150 and titanium-coated BCP1150Ti possessing small surface microstructure (~1 μm) formed ectopic bone adjacent to multinucleated osteoclast-cells in the muscle of dogs. Implants were in the form of planar discs so macro-scale features such as concavities, macropores and interparticle space were unnecessary for this response. In contrast, BCP1300 with identical compositional chemistry but larger surface architecture (~2-4 μm) formed neither osteoclast-like cells nor ectopic bone; it was instead encapsulated by fibrous tissue. Similar to the in vitro results, (pre-)osteoclast proliferation and differentiation were significantly promoted by BCP1150 and BCP1150Ti versus BCP1300 in vitro; moreover, osteoclasts were larger and more fused on BCP1150 versus either BCP1150Ti or BCP1300. Together, these in vitro and in vivo results indicate that (sub)micron-scale surface architecture is the crucial material parameter versus macrostructure or surface chemistry in stimulating both osteoclastogenesis and ectopic bone formation in a related process.

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Editor’s Note: All questions/comments by the reviewers were answered by text changes. There is hence no Discussion with Reviewers section.