Development of a new bone-mimetic surface treatment platform: Nano-needle hydroxyapatite (nnHA) coating

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1 Abstract

The hierarchical structure of bone plays pivotal roles in driving cell behaviour and tissue regeneration and must be considered when designing materials for orthopaedic applications. Herein, we aim to recapitulate the native bone environment by using melt electrowriting (MEW) to fabricate fibrous micro-architectures which are modified with plate shaped (pHA) or novel nano-needle shaped (nnHA) crystals. Nuclear magnetic resonance spectroscopy, SEM, TEM and x-ray diffraction demonstrated that these coatings replicate the nano-structure and composition of native bone. Human mesenchymal stem/stromal cell (MSC) mineralisation was significantly increased 5-fold with pHA scaffolds and 14-fold with nnHA scaffolds. Given the protein stabilising properties of mineral, these materials were further functionalised with BMP2. nnHA treatment facilitated controlled release of BMP2 which further enhanced MSC mineral deposition. Finally, we have demonstrated the versatility of this nnHA treatment method, which may be used to coat different architectures/materials including fused deposition modelling (FDM) scaffolds and Ti6Al4V titanium. This study thus outlines a method for fabricating scaffolds with precise fibrous micro-architectures and bone mimetic nnHA extrafibrillar coatings which significantly enhance MSC osteogenesis and therapeutic protein delivery, and leverages these results to show how this surface treatment method may be applied to a wider field for multiple orthopaedic applications.

Keywords: Melt electrospinning writing; BMP-2; Osteogenesis; Tissue Engineering; Orthopaedics;

2 Introduction

Recapitulating the mineral environment of bone is of great relevance for orthopaedic medicine, from the successful osteointegration of biomaterials and implants, to the development of regenerative

scaffolds for treating bone injuries and diseases. Autologous tissue is the current gold standard for bone repair due to its osteoconductive, osteoinductive and immunocompatible properties, however, there are well documented drawbacks to its widespread clinical use [1]. Due to technology advancements and an improved understanding of the physiological mechanisms of bone repair, synthetic tissue engineering approaches have demonstrated great progress in developing bioinspired materials that can promote bone formation [2]. These approaches continue to fall short of the gold standard however, therefore, continued development of synthetic materials to guide bone formation/healing is required. To achieve this, it is important to understand the structure of native bone. Bone is a complex, hierarchical multi-phasic organ comprised primarily of mineralised collagen fibrils spanned by additional continuous, cross-fibrillar mineral units [3, 4]. The precise fundamental structure of bone remains unclear, however, it is known that it comprises of a plate-like apatite structure which likely self-assembles into organised structures [5], with crystals being preferentially aligned with the collagen fibrils [6]. It has also recently been shown that the mineral structure of bone may be neither exclusively platelet nor needle shaped [4]. Needle-shaped mineral units of base 5 nm and length 50 – 100 nm may comprise a more fundamental component of the bone nanostructure, with these needles merging to form platelets and mineral stacks and aggregate to form irregular 3D structures of size 200 – 300 nm which are crossfibrillar in nature [4]. Nonetheless, it is the combination of collagen and mineral with specific topographies, working in unison in this hierarchical structure, which gives the bone both its excellent biological and mechanical properties. Moreover, the mineral nano-topography identified in bone is believed to be a key contributor to non-collagenous protein binding and stabilisation, preserving proteins for centuries within the tissue without denaturation [7]. This therefore provides a potential strategy for therapeutic delivery in regenerative medicine [8]. Development of unique multiscale architectures which recapitulate the nanostructure of this tissue may represent an innovative approach to develop effective bioinspired materials to promote bone repair.

The composite fibrous structure of bone contributes to its unique structural properties. During intramembranous ossification and bone remodelling, osteoblasts originating from mesenchymal progenitor cells lay down collagen fibrils and enrich the surrounding environment with non-collagenous proteins and proteoglycans to promote mineral deposition within the collagen template [9-12]. The resulting nano-crystals are initially formed within collagen fibrils, eventually nucleating to fill the voids forming a composite structure of mineral and collagen fibres approximately 1 - 10 μ m in diameter, which in turn are composed of nanoscale collagen fibrils [13, 14]. The underlying collagen fibrillar template is thus crucial in dictating cellular behaviour in bone and many other tissues, with dysregulation of this unique structure leading to undesirable outcomes including cell death, abnormal

differentiation, tumour development and diseases such as osteoporosis and osteogenesis imperfecta [15, 16]. Through the advent of new technology such as 3D printing, precise control of scaffold architecture is now achievable [17]. Fused deposition modelling (FDM) can produce scaffolds with defined fibre architecture creating pores of differing shapes [18] in addition to facilitating mineral incorporation [19-23], which in turn drives mesenchymal stem/stromal cell (MSC) differentiation down specific lineages. However, FDM is limited in that fibre diameters below 100 μm are difficult to fabricate. These relatively large fibres result in a pseudo-3D environment at the scale of the cell [24]. Electrospinning is a common fabrication technique to produce physiological fibre diameters on the nano/micron scale which have been utilised in bone tissue engineering strategies [25, 26]. However, due to the unstable “whipping” motion of the electrospinning jet during fibre deposition, there is limited control over fibre architecture using this approach. Melt electrowriting (MEW) is a recently developed 3D printing technology which overcomes the above limitations of FDM and electrospinning, being capable of fabricating and controlling the deposition of micron-scale fibres [27-29]. This facilitates the manufacture of complex fibrous micro-architectures [30-34] consistent with that seen in native extracellular matrix (ECM) including that of bone. We recently utilised MEW to fabricate scaffolds with bone inspired fibre diameters of 10 μm and investigated whether the fibre architecture in terms of degrees of alignment could influence stem/stromal cell behaviour [35]. Interestingly, the combination of fibre diameter and alignment had significant impact on cell shape, cell signalling and ultimately MSC osteogenesis. A 90° fibre architecture drove a 4-fold increase in stem/stromal cell mineralization when compared to 10° aligned architectures or traditional random electrospun scaffolds. This demonstrates the critical role of fibrillar architecture in mediating stem/stromal cell behaviour and highlighting MEW as a powerful tool to fabricate physiological bioinspired environments for tissue engineering strategies.

In bone tissue, the mineral component makes up 60 - 70 % of its dry weight, and is composed primarily of non-stoichiometric apatite along with other loosely bound ions including Ca^{2+} , HPO_4^{2-} , CO_3^{2-} and Mg^{2+} in a hydrated layer at the surface [36-38]. As with fibrillar architecture, the mineral structure in bone plays a key role in mediating cell behaviour, with deficiencies or changes in mineral composition contributing to diseases such as osteoporosis [39]. This has been exploited in a host of strategies for orthopaedic and dental applications, where materials and surfaces have been modified with calcium phosphates with the aim of creating biomimetic materials which promote osseointegration and tissue regeneration. This includes coatings for implant fixation, cements and adhesives for orthopaedic and dental implants, and synthetic bone grafts [40]. A wide range of commercially available materials have been investigated, including hydroxyapatite (HA), calcium deficient apatite (CDA), α - and β tricalcium phosphate (TCP) and biphasic calcium phosphates such as HA + TCP, with a range of other materials

including dicalcium phosphate (DCP) and Tetracalcium phosphate (TTCP) also being investigated [41]. As with fibrillar architecture, an ever increasing appreciation for the importance of the architecture and particle size of the mineral phase is being gained, which defines surface topography and thus has a considerable influence on cell behaviour and implant performance [42]. When cultured on nano-scale HA (nHA) films with varying particles sizes from 20– 80 nm in diameter, MSCs exhibit a trend of greater viability and proliferation with smaller particle sizes [43] while another study has demonstrated that HA nanotopography with a grain size of 104.6 nm enhanced MSC osteogenesis *in vitro* and osteoinduction *in vivo* [44]. The osteogenic capacity of nHA has been further demonstrated in a range of studies where cells have been cultured in nHA suspensions with particles of size between 20 – 100 nm, with cells demonstrating uptake of particles and exhibiting enhanced osteogenic differentiation [45-47]. Appropriately sized nHA particles can also preferentially polarize human macrophages towards an M2 phenotype and enhance production of the anti-inflammatory cytokines such as IL-10 [48]. The role of HA particle size has also been investigated in the 3D printing of composite polycaprolactone-HA (PCL-HA) blends, with enhanced proliferation and alkaline phosphatase (ALP) activity of MSCs seen with nano-particles compared to micro-particles [49]. Remarkably, particle size has not only been shown to be an important cue for MSC osteogenesis, but also plays a role in suppressing undesired cellular outcomes in bone. This has been demonstrated via osteosarcoma inhibition [43] and reduced melanoma proliferation [50] with nHA compared to larger particles, further reinforcing the therapeutic potential of nano-scale particles for additional applications such as bone regeneration following tumour ablation. A recent study has also demonstrated the regenerative capacity of grafts with a needle surface topography *in-vivo*, with sub-micron needles triggering earlier and accelerated bone formation [51].

Bone tissue also houses non-collagenous proteins and growth factors embedded within its composite matrix, which are remarkably resolute and can be preserved for centuries due to the great stability provided by the mineral structure [7]. It is thus of no surprise that the great binding affinity to HA of a host of biological and synthetic components has been exploited for therapeutic benefits. HA has been extensively used as a delivery mechanism for bone morphogenetic protein 2 (BMP2) protein with proven success in a number of in-vivo models [52-54], while nHA particles have also been used for non-viral gene delivery of BMP2 to enhance MSC osteogenesis [55, 56]. Anti-tumour drugs have also been combined with nHA as a delivery vector, with potential applications in osteosarcoma chemotherapy [57] and the development of targeted drug delivery methods [58]. Interestingly, protein binding and stabilization is closely related to the nanoscale structural features of the mineral coatings [8], with a mineral nanotopography facilitating maintained conformation of BMP2 and enhanced cell adhesion and osteogenesis [44]. In addition to the already pro-osteogenic capabilities

of biomimetic nHA, there is thus great potential to exploit its natural affinity to further advance treatments by facilitating therapeutic delivery providing an extensive range of enhanced regenerative benefits.

In summary, mineral composition and architecture play fundamental roles in bone biology. While mineral coatings are commonly used for biomedical and tissue engineering applications, an appreciation for their architecture, composition and how these factors influence biological performance is lacking. Thus, in this study, we develop and characterise a new nano-needle hydroxyapatite (nnHA) surface treatment method which may be used to coat materials with a nano-scale HA mineral layer inspired by the nano-architecture and composition of bone. We use MEW to fabricate fibrous 3D PCL micro-architectures with 90° fibre orientations and 10 µm fibres which we have previously shown to be significantly pro-osteogenic in comparison to other studied architectures [35]. We then study the developed nnHA surface treatment using MEW as scaffold platform and compare it to three other groups: uncoated fibres, a commonly used SBF precipitation protocol resulting in HA with a plate morphology (pHA) and fibres with intrafibrillarly dispersed nHA (iHA). We extensively characterise the developed nnHA coating in addition to other groups, undergoing a range of material and biological characterisation methods. Fibre surface topography was significantly altered with the above treatments, with both the pHA and nnHA groups found to be comprised of mineral units with a crystalline HA core and amorphous surface layer analogous to the structure of native bone mineral. Subsequent biological characterisation with hMSCs demonstrated a significant upregulation of cell deposited mineral in the nnHA group, while this group was also shown to facilitate BMP-2 loading, sustained release and enhanced hMSC osteogenesis, indicating its potential as a vector for factor delivery. Finally, we demonstrate the versatility of the developed nnHA surface treatment approach, which we have shown here can be used to form identical mineral coating layers on FDM scaffolds and Ti6Al4V titanium alloys. This versatility, the simplicity of the protocol and its osteogenic capacity make it an attractive method for modifying pre-made constructs for applications in bone tissue engineering, biomedical device coating and the study of the microenvironment of bone.

Figure 1. Outline of material development and characterisation. **A** Control (C) scaffolds were fabricated via MEW and subsequently coated with HA plates (pHA) using an established protocol and HA nano-needles (nnHA) using a new protocol developed within this study. **B** Scaffolds were also fabricated with HA loaded PCL (iHA). **C** Materials and scaffolds were analysed using a range of characterisation methods, and subsequent biological characterisation was conducted using human MSCs.

3 Materials and methods

3.1 Scaffold fabrication

3.1.1 Polycaprolactone (PCL) melt electrowriting

Fibrous scaffolds with a fibre diameter of 10 μm , square apparent pore size of 50 μm , and layer offset of 300 μm were fabricated on a custom built MEW apparatus as previously described [35]. We have demonstrated that this fibrous scaffold architecture drives MSC osteogenesis [35]. Briefly, heated air at a temperature of 90 $^{\circ}\text{C}$ was circulated around a syringe to melt polycaprolactone (PCL) (Sigma Aldrich 440744, average Mn 80 kDa), with air pressure being used to extrude the polymer through a 21G needle with high voltage applied at a distance of 15 mm from a grounded aluminium collector plate. Fibres were deposited with a 90 $^{\circ}$ offset between subsequent layers to result in a square pore shape. Scaffolds for control (C) groups were used without further mineral modification.

3.1.2 Plate hydroxyapatite (pHA) coating

This coating was carried out via a saturated simulated body fluid (SBF) solution, as described by Martine *et al* [59]. Reactions were carried out in 50 mL conical tubes, with reagent volumes being maintained at 40 mL at each step of the process and 6 scaffolds of dimension (3 x 3) cm being processed per tube. Scaffolds were immersed in 70 % ethanol at room temperature for 15 min and a vacuum applied for the entire duration to remove air bubbles. Scaffolds were then placed in 2M NaOH

under vacuum for 5 min to remove air bubbles, and then incubated in tubes in a water bath at 37 °C for 45 min. Scaffolds were then rinsed 5 times in MilliQ water. 10X SBF solution was brought to a pH of 6 and scaffolds were placed in the solution and a vacuum was applied for 5 min. Scaffolds in tubes were then incubated in a water bath at 37 °C for 30 min. The coating step in 10x SBF was repeated a further two times as above with fresh solution minus the vacuum treatment, for a total of three mineral coatings. Scaffolds were then treated with 0.5M NaOH for 30 min at 37 °C, rinsed 5 times in MilliQ water and allowed to dry overnight. Scaffolds treated with this coating method are defined as plate hydroxyapatite (pHA).

3.1.3 Nano-needle hydroxyapatite (nnHA) coating

We developed a nano-hydroxyapatite coating to coat scaffolds with nano-needle mineral units. Sample pre- and post-processing was carried out as per the calcium phosphate coating procedure, with the 10x SBF treatment step being substituted for alternative calcium and phosphate solutions [60, 61], with the entire procedure outlined as follows. Reactions were carried out in 50 mL conical tubes, with reagent volumes being maintained at 40 mL at each step of the process and 6 scaffolds of dimension (3 x 3) cm being processed per tube. Scaffolds were immersed in 70 % ethanol at room temperature for 15 min and a vacuum applied for the entire duration to remove air bubbles. Scaffolds were then placed in 2M NaOH under vacuum for 5 min to remove air bubbles, and then incubated in tubes in a water bath at 37 °C for 45 min. Scaffolds were then rinsed 5 times in MilliQ water. Calcium and phosphorus solutions were developed to achieve a homogenous mineral coating. The calcium solution was made with 0.05M calcium chloride dihydrate (Sigma Aldrich C7902) in MilliQ water. The phosphorus solution was made with 0.03M sodium phosphate tribasic dodecahydrate (Sigma Aldrich S7778) and 0.01M sodium hydroxide in MilliQ water. These solutions were stored at 4 °C for up to 1 month. Scaffolds were rinsed 5 times in MilliQ water and added to 20 mL of the calcium solution. 20 mL of the phosphorus solution was slowly added to the calcium solution, a vacuum applied for 5 min to remove air bubbles, and scaffolds were then incubated for 30 min in tubes at 37 °C in a water bath. This coating procedure was repeated a further two times minus the vacuum treatment, with fresh calcium and phosphorus solutions being used each time, resulting in a total of 3 mineral coatings. Scaffolds were then treated with 0.5M NaOH for 30 min at 37 °C, rinsed 5 times in MilliQ water and allowed to dry overnight. Scaffolds treated with this coating method are defined as nano-needle hydroxyapatite (nnHA).

3.1.4 Hydroxyapatite incorporated (iHA) PCL melt electrowriting

A nHA-PCL blend was created by incorporating a 3.5wt% of nano-hydroxyapatite (Sigma Aldrich 677418) into the polymer. PCL and nano-hydroxyapatite with a total mass of 1 g was added to a glass

vial, with 10 mL of chloroform being added under stirring until dissolved. Particles were dispersed in the solution by ultrasonication for 1 h, after which it was poured into a glass dish and left overnight for chloroform to evaporate. The resulting film was formed into pellets which were loaded into 1 mL BD-Braun syringes. The syringes were heated for 1 h at 110 °C, and centrifuged at 4,000g for 60 s to remove air. The previous heating and centrifugation steps were repeated for a total of 8 cycles, or until a homogenous blend was present in the syringe with no air bubbles. MEW processing parameters were optimised to facilitate controlled deposition of PCL-nHA blends, with temperature increased to 110 °C and voltage increased by approximately 0.5 kV. Scaffolds for this modification method are defined as fibre incorporated hydroxyapatite (iHA). To verify that sedimentation of nHA particles did not occur as a result of this process and during MEW printing, a scaffold was fabricated for 1 h, fibres were then allowed to deposit at the edge of the print plate for 3 h, and finally, a second scaffold was fabricated for 1 h. SEM imaging was conducted on each scaffold to assess the homogeneity of nHA within fibres. Additionally, a thermogravimetric analysis (TGA) was conducted on 10 mg of material extruded from the syringe to assess the mineral content.

3.2 Scaffold and mineral characterisation

3.2.1 SEM imaging and Energy-dispersive X-ray spectroscopy

For SEM imaging, samples were prepared for imaging by coating with gold/palladium for 40 s at a current of 40 mA. Imaging was conducted in a Zeiss ULTRA plus SEM using InLens and SE2 detectors with an accelerating voltage of 4– 5 kV. For analysis with energy-dispersive X-ray spectroscopy (EDX), scaffolds were carbon coated and analysed at an accelerating voltage of 15 kV in a Zeiss ULTRA plus SEM with an 80 mm² Oxford Inca EDX detector. To investigate approximate calcium/phosphorus atomic ratios for each group, spectra were acquired on scaffolds for 30 s (n=6, 2 technical replicates per scaffold). Element maps were constructed by taking 2 frames at a resolution of 256 x 192 with map dwell of 4000 µs and linescan dwell of 2000 µs.

3.2.2 TEM imaging

To collect mineral from pHA and nnHA groups for TEM imaging, coated scaffolds were submerged in 100 µL anhydrous ethanol in microtubes and ultrasonicated for 30 min. For the iHA group, powder was added directly to ethanol and ultrasonicated for 30 min to disperse particles. Approximately 5 µL of each sample was loaded on to Lacey carbon coated copper TEM grids (Agar Scientific, UK) and allowed to dry overnight. Samples were imaged on a JEOL JEM-2100 electron microscope operating at 200 kV.

3.2.3 X-ray diffraction

To obtain powder samples for x-ray diffraction (XRD) analysis, 10x SBF was allowed to precipitate in solution at a pH of 6 for the pHA group. For the nnHA group, calcium and phosphate solutions were mixed together and allowed to precipitate. Solutions were centrifuged at 5,000g for 40 min to collect the precipitate, which was then placed in dishes and dried for 4 h at 50 °C in an oven. The resulting mineral was ground into a powder using a mortar and pestle. The hydroxyapatite powder used for the iHA group was used for XRD analysis without any further processing. Samples were loaded in a Bruker D8 ADVANCE powder diffractometer and run for 1 h from 5 - 80° in the 2θ range with a monochromatic Cu-Kα radiation source. Values for crystallinity were estimated using DIFFRAC.SUITE EVA 4.1.1. Crystallite size was calculated from taking 5 measurements of the (211) peak in pHA and iHA groups. In the nnHA group, crystallite size was calculated from the peak in the position of (211), (112) and (300), due to the broad peaks resulting in their merging.

3.2.4 Nuclear Magnetic Resonance (NMR) spectroscopy

Solid-state Nuclear Magnetic Resonance (ssNMR) spectroscopy experiments were conducted on a Bruker 400 MHz Avance-III spectrometer running TopSpin software. For pHA and nnHA groups, (12 x 8) mm diameter coated MEW scaffolds were packed into 3.2 mm (outer diameter) zirconia rotors. The commercially available powder used in the iHA group was directly added to zirconia rotors for analysis. NMR spectroscopy of a native bone control sample was conducted on a cortical bone tissue sample was harvested from a healthy 2-year-old sheep, which was extracted from the distal femoral metaphysis. Zirconia rotors were spun at a Magic Angle Spinning (MAS) frequency of 20 kHz. ¹H chemical shifts were referenced to TetraMethylSilane (TMS) at δ¹H = 0.0 ppm, whereas ³¹P chemical shifts were referenced to H₃PO₄ (85 % w/w aqueous solution) at δ³¹P = 0.0 ppm.

For 1D ¹³C single-pulse (SP) MAS ssNMR experiments (Figure 3A), the number of scans (NS) for samples were as follows: [bone tissue, NS = 40], [pHA, NS = 240], [nnHA, NS = 240], [iHA powder, NS = 16], with a recycling delay for 300 s for each. For 2D {¹H}³¹P Heteronuclear Correlation (HetCor) MAS ssNMR experiments (Figure 3B-D), NS for samples was as follows: [bone tissue, NS = 128 in each 260 t1 increments], [pHA, NS = 128 in each 260 t1 increments], [iHA powder, NS = 128 in each 384 t1 increments]. A contact time (t_{CP} = 1 ms) and recycling delay (RD = 4 s) was used for each. To create illustrate non-normalized, individual ³¹P NMR signals of both the apatitic core and the amorphous surface layer (Figure 3E-G), individual ³¹P NMR signals were extracted from the 2D {¹H}³¹P HetCor MAS ssNMR spectra. Specifically, the sums of the F2 slices taken at the OH⁻ ions position in F1 (blue areas in Figure 3B-D) have been used to generate the individual ³¹P signals of the crystalline nanoparticle core in the form of hydroxyapatite. Further, the sums of the F2 slices taken at the H₂O molecules and

HPO₄²⁻ ions positions in F1 (orange areas in Figure 3B-C) have been used to generate the individual ³¹P signals of the amorphous surface layer. The molar ratio of PO₄³⁻ and HPO₄²⁻ ions, respectively present in the apatitic crystalline core and the amorphous surface layer belonging to the hydroxyapatite nanoparticles associated with pHA and nnHA MEW scaffolds, was estimated by fitting their respective ³¹P single pulse (SP) MAS ssNMR spectra which were recorded in quantitative conditions (RD = 300 s, NS = 240). In the case of the pHA MEW scaffold, both the lineshape and the linewidth of these two peaks are known, and as such, their respective chemical shift and FWHM are known and these values were held constant, while only the peaks intensities were set free during the fit. In contrast, the lineshape and the linewidth of these two peaks could not be experimentally determined in the case of nnHA MEW scaffold due to the absence its 2D {¹H}³¹P HetCor MAS ssNMR spectrum which could not be recorded due to the relatively low volume of mineral present. As a result, the chemical shift, FWHM and peaks intensities were all estimated according to those determined from the pHA MEW scaffold. Last, the area under each peak was integrated to obtain the molar ratio of PO₄³⁻ and HPO₄²⁻ ions respectively present in the apatitic crystalline core and the amorphous surface layer.

3.2.5 Calcium staining

Samples were taken from scaffolds using a 2 mm biopsy punch (n=5) and incubated with 1 % alizarin red s (Sigma Aldrich, A5533) in distilled water for 10 min at a pH of between 4.1 – 4.3. Samples were rinsed 3 times with water and allowed to dry prior to imaging. To quantify bound stain, 400 µL of 10 % acetic acid was applied and samples incubated at room temperature for 30 min while shaking at 150 rpm. The acetic acid was added to 1.5 mL tubes, vortexed vigorously and heated to 85 °C for 10 min. Tubes were transferred to ice for 5 min, centrifuged at 20,000g for 15 min, and 300 µL of the supernatant was added to new tubes along with 120 µL of 10 % ammonium hydroxide. Standards were made with dilutions of alizarin red solution in distilled water, with the pH of each adjusted to between 4.1 – 4.3. Samples and standards were read at 405 nm in a 96-well plate.

3.2.6 Surface roughness evaluation via white light interferometry

The surface topography of pHA and nnHA coatings in addition to uncoated controls was investigated using a Filmetrics Profilm 3D white light interferometer using 113.10 x 84.46 µm analysis regions on flat Ti6Al4V specimens (n=3 per sample). For each sample and analysis region, surface roughness (Ra, ISO 4287) was determined at 9 horizontal line profiles at a spacing of 10.25 µm.

3.2.7 Water contact angle

The water contact angle of all scaffold groups was quantified using a FTA125 contact angle analyzer (First Ten Angstroms Inc.). Samples were prepared using an 8 mm biopsy punch (n=6-7). All measurements were taken 10 frames after contact of the water with the sample.

3.2.8 Tensile testing and coating stability

Rectangular samples (n=4) of dimension (5 x 20) mm were taken from scaffolds and used for tensile testing in a Zwick Z005 with 5 N load cell (A.S.T. – Angewandte System Technik GmbH). Samples were pre-loaded to 0.002 N at a speed of 1 mm/min, and then loaded at a speed of 10 mm/min with a time save interval of 0.1 s. Load-displacement curves were graphed and used to determine the stiffness, toe region stiffness and yield force for each group.

The mechanical stability of coatings was assessed by applying either 1 N or 10 N of force on nnHA coated FDM fabricated scaffolds of diameter 8 mm. Scaffold were then drawn a distance of 100 mm at a speed of 20 mm/s on either flat aluminium or latex surfaces to simulate implantation or handling respectively. A brushing force was also applied to another group of scaffolds. Scaffolds were imaged using SEM to assess coating damage from the above mechanical load regimes.

3.3 Human bone marrow mesenchymal stem/stromal (hMSC) cell culture

Scaffolds were punched to a diameter of 8 mm and UV sterilised for 20 min on each side before being mounted in stainless steel holders and placed in 24-well plates. Scaffolds were then pre-wet in a graded ethanol series of 100 %, 90 % and 70 % for 20 min each before being washed three times in phosphate buffered saline (PBS). They were then incubated overnight in DMEM with 10 % FBS at 37 °C. Human bone marrow mesenchymal stem/stromal cells (hMSCs) were isolated from bone marrow (Lonza, US), trilineage potential verified [62], and seeded at a number of 10,000 cells per scaffold. Scaffolds were transferred to new well plates after 24 h, and cultured in osteogenic medium (OM) from day 3, which consisted of 10 % FBS DMEM supplemented with 100 nM dexamethasone, 10 mM β -glycerol phosphate and 50 μ g/ml ascorbic acid. Medium was changed every 3.5 days.

3.4 Proliferation

At days 1,7,14 and 21, scaffolds were added to 100 μ L lysis buffer (n=4) containing 0.2 % Triton X-100, 1 mM Tris pH8, with phenylmethylsulfonyl fluoride (PMSF) being added at a ratio of 1:200 just before use, and stored at -80 °C. Before DNA quantification, samples underwent ultrasonication for 60 s and subjected to three freeze-thaw cycles in liquid nitrogen before being stored on ice. DNA content was quantified using a Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen, P7589), with excitation and emission wavelengths of 485 nm and 528 nm respectively. The DNA content in 10,000 hSSCs seeded and pelleted in centrifuge tubes was also quantified to calculate the total number of cells present on scaffolds.

3.5 Characterisation of hMSC osteogenic differentiation

3.5.1 Intracellular alkaline phosphatase (ALP)

Intracellular ALP was quantified at days 14 and 21 (n=4). 50 µL of 5mM pNPP was added to wells along with 10 µL of cell lysate and 70 µL MilliQ water in a 96-well plate. Standards were comprised of serial dilutions of p-Nitrophenyl phosphate (pNPP, Sigma Aldrich, N1891) with 10 µL of 43 µM ALP enzyme (Sigma Aldrich, P6774) being added to each. Plates were incubated for 1 h in the dark at room temperature, and reactions were then stopped using 20 µL of 3M NaOH. Absorbance was read on plates at 405 nm, and ALP activity was calculated as the amount of pNPP generated as a function of sample volume and reaction time.

3.5.2 Collagen production

Scaffolds were cultured up to 21 days before evaluating collagen content. Cell-scaffold constructs were rinsed in PBS, fixed in 10 % neutral buffered formalin for 15 min and rinsed again in PBS before storage at -20 °C. Scaffolds were stained with 200 µL of 1mg/ml of Direct Red 80 (Sigma Aldrich, 365548) in a saturated aqueous picric acid solution for 1 h with shaking at 150 rpm. Scaffolds were then washed twice with 0.5 % acetic acid and allowed to dry overnight before imaging. To quantify collagen content, 500 µL 0.5M NaOH was added to wells under shaking until stain was dissolved, and solutions were added to 1.5 mL tubes. Tubes were centrifuged at 14,000g for 10 min to pellet debris. Standards were made by adding direct red staining solution to 8 µL of collagen I (Corning, #354249) before centrifuging at 14,000g for 10 min and re-suspending the collagen in 500 µL 0.5M NaOH. The absorbance of samples and standards were read at 490 nm in 96-well plates.

3.5.3 Calcium production

Cell-scaffold constructs were investigated for total calcium content after 21 days using 1 % alizarin red S solution as previously described for calcium staining in scaffold characterisation. Cell-free scaffolds were also cultured up to 21 days to determine the contribution of total calcium from cell mineralisation versus the calcium present due to mineral modification of scaffolds.

3.6 BMP2 functionalisation of MEW scaffolds

3.6.1 BMP2 adsorption onto scaffolds

Scaffolds were prepared, sterilised and pre-wet in a graded ethanol series as previously described in section 3.3 before being washed three times in PBS. Recombinant human BMP2 (Peprotech 120-02) was diluted to 50 µg/mL in PBS, with 20 µL of solution containing a total of 1 µg BMP2 being placed

on each scaffold and incubated for 4 h at room temperature. BMP2 was then removed and scaffolds were allowed to dry overnight.

3.6.2 BMP2 release kinetics

Scaffolds were placed in 48-well plates (n=4) and 200 µL of PBS added to each. At each time-point, PBS was removed and 1 % BSA added to result in a final BSA concentration of 0.1 %. 200 µL of fresh PBS was then added to each well. Samples were stored at -80 °C until BMP2 quantity was determined via ELISA (R&D Systems DY355-05). Cumulative BMP2 release was studied at time-points 1 h, 6 h, and days 1, 3, 7, 14 and 21.

3.6.3 Characterisation of osteogenic differentiation

After BMP2 adsorption, 10,000 hMSCs were seeded onto scaffolds (n=4), and cultured up to 21 days after which DNA content was investigated to determine total cell number. Scaffolds were also cultured up to 21 days to assess osteogenic potential in terms of ALP activity, collagen production and calcium production as described previously.

3.7 Investigating the versatility of nnHA surface treatment

It has previously been shown that SBF treatment may be used to treat a range of materials with a spherulitic/plate based coating [63-66]. We validated this with the pHA coating protocol used in the present study and subsequently investigated if the nnHA coating could be applied to other geometries/materials.

FDM scaffolds were design with a (0 – 90)° orthogonal fibre pattern using the BioCAD™ software package. These scaffolds were fabricated using the melt extrusion printhead on the 3D Discovery multi-head bioprinting system (Regen Hu, Switzerland). Briefly, PCL (PCL; CAPA Ingevity, SC, USA, average Mn 50 kDa) was extruded through a 32G needle at a temperature of 85 °C, the resulting fibres were 131.32 µm (SD = 4.74) in diameter with an apparent fibre-fibre spacing of 746.51 µm (SD = 8.26). Samples of Ti6Al4V titanium were also prepared for coating by cutting them in to pieces of approximately (5 x 100) mm. The above FDM and titanium samples, in addition to MEW scaffolds fabricated with the previously used PCL (Mn = 80 kDa) were treated as previously for pHA and nnHA coatings. In addition, it was investigated if the nnHA coating thickness could be increased by changing the total number of coating steps from 3 to 12.

3.8 Statistical analysis

Tensile testing and material characterisation data is presented in terms of average and standard deviation. Subsequent biological data is presented in terms of average and standard error of the mean. Statistical analysis was performed using one-way and two-way ANOVA and Tukey's multiple comparison post-test unless otherwise stated.

4 Results

4.1 MEW fibre topography is significantly altered by bulk and surface modification

The topography of scaffold treatments were investigated via SEM and TEM imaging, where significant changes were identified with minimal influence on macroscopic scaffold morphology. Uncoated control ('C') fibres have a diameter of 9.09 μm (SD = 1.00), with relatively featureless surface topography (Figure 2A, Table S 1). The plate hydroxyapatite (pHA) coating protocol yields a spherulitic/rosette-like arrangement of plates as seen by measuring grain size via SEM (width = 555.30 nm, thickness = 25.98 nm). These in turn are composed of smaller crystallites (width = 142.15 nm, thickness = 10.07 nm) as seen by TEM (Figure 2B,H). Total fibre diameter including the coating is 13.53 μm (SD = 2.63), giving an average coating thickness in the pHA group of 2.22 μm (Table S 1). In stark contrast to this, the nano-needle hydroxyapatite (nnHA) coating protocol results in the formation of nano-needles on the fibre surface (Figure 2C,H). These needle-structured mineral units have a grain size which is approximately 100 nm in length with a thickness of 37.04 nm as imaged by SEM, and are seen to form occasional aggregates which range in diameter from 150 – 500 nm. TEM imaging reveals a smaller crystallite unit with length 58.19 nm and thickness 10.42 nm. Total fibre diameter shows a minimal increase compared to the control with a mean value of 10.19 μm (SD = 1.02), yielding a total coating thickness in this group of 0.55 μm (Table S 1). The incorporation of hydroxyapatite within fibres (iHA) during the MEW process yields minimal changes in surface topography, with the majority of visible particles being seen as nodules just below the fibre surface, while several can also be seen to protrude above the surface with no covering of PCL (Figure 2D). iHA particles/aggregates have an average diameter of 147.17 nm as measured within PCL fibres via SEM, while individual grains have an average diameter of 89.85 nm and median diameter of 69.84 nm as measured using TEM imaging (Figure 2E-G). A summary of measurements is illustrated in Figure 2H, with typical morphology and median measurements in Figure 2I-K. The incorporation of HA also has an influence on the consistency of the MEW printing process, with periodic pulsing of the Taylor cone due to the presence of particles within the melt being difficult to control, resulting in a larger fibre diameter distribution (fibre diameter = 11.79 μm , SD = 4.19) (Table S 1). Sedimentation was not found to be an issue when printing

with the iHA material, as demonstrated by similar mineral distribution when using material at both the beginning (Figure S 1A) and end (Figure S 1B) of the syringe stock. Additionally, the total mineral content of a printed MEW scaffold was verified to be 3.45% (Figure S 1C), similar to the 3.5wt% of the initially synthesised material. White light interferometry was used to estimate the surface roughness of coatings, with the pHA coating ($R_a = 0.29 \mu\text{m}$) having no effect on surface roughness compared to the control ($R_a = 0.30 \mu\text{m}$), while nnHA coating yielded a roughness of $R_a = 0.40 \mu\text{m}$ (Figure S 3). Due to the low thickness of the latter coating, the roughness of the underlying structure will likely remain a factor in final surface roughness after coating with nnHA.

The levels of calcium on the surface of each scaffold was estimated *via* quantification of alizarin red staining. Minimal calcium was detected on the control scaffolds, while the greatest amount of calcium was found on pHA coated scaffolds (Figure 2L). The nnHA group contained the second highest level of calcium, but was still 6-fold less than the pHA groups and was not significantly different to the iHA group. The calcium content detected in iHA scaffolds is only marginally greater than that detected from background staining (C) due to the majority of HA particles being distributed throughout the bulk of the fibres.

457 **Figure 2 Electron microscopy of scaffold micro-architecture and mineral nano-structure.** A-D SEM
458 imaging of scaffold groups C, pHA, nnHA and iHA respectively, illustrating the “plate” coating
459 morphology in pHA, “needle” morphology in nnHA, and the presence of HA nanoparticles both
460 within and protruding above the surface of the fibres in iHA scaffolds. E-G TEM imaging of mineral
461 units of pHA, nnHA and iHA groups respectively, confirming their morphology. H Mineral size
462 measurements as measured via SEM and TEM with I-K Typical particle morphology and median
463 dimensions. L Quantification of total calcium content in scaffold groups (n=5).

4.2 CaP treatments modify surface chemistry

4.2.1 Element analysis

The coating chemistry and distribution in fibres was investigated via EDX analysis. The presence and distribution of calcium and phosphorous was first investigated via element mapping where, as expected, there was no detection in the control (Figure S 2A). A consistent distribution of both elements was seen in the pHA group (Figure S 2B) and nnHA (Figure S 2C) coated fibres, while a sparse distribution in the iHA group (Figure S 2D) confirmed the presence of HA particles within fibres. Carbon and oxygen were present in all groups due to contributions from the PCL, as well as calcium and phosphorous as seen in the spectra of all groups except the control. A key difference in the pHA group was seen with the presence of sodium and magnesium in this coating, with these being components of the 10x SBF solution used for precipitation. While sodium and chlorine among others are components of the nnHA coating solutions, these were not components of the precipitated coating as seen by their absence in the EDX spectra.

4.2.2 Crystal structure of surface and bulk modifications

XRD analysis was further conducted to characterise the crystal structure of the different groups (Figure S 4). Brushite, hydroxyapatite and halite were identified in the pHA group, in close agreement with previous literature which used this coating approach [67], with an estimated crystallinity of 61.3 %. The nnHA and iHA groups were both shown to be composed solely of hydroxyapatite, with crystallinity of 52.5 % and 69.3 % respectively. The broader peaks in the nnHA group are indicative of a smaller crystallite size, which was found to be 8.5 nm. This is in comparison to the larger crystallite sizes in the pHA and iHA groups, which were measured at 105.8 nm and 75.0 nm respectively, in close agreement with TEM measurements. Note that XRD analysis was conducted on the entire collected precipitate which was collected via centrifugation, as sufficient quantity of mineral could not be obtained from coated samples. Thus, this analysis may not be completely representative of the resulting mineral coating present on scaffolds. Further characterisation on coated scaffolds was thus conducted to further understand their molecular structure via NMR as outlined below.

4.2.3 Coated scaffold groups contain a crystalline HA core and bone-mimetic amorphous surface layer

ssNMR was applied to analyse the calcium phosphate solids associated with the mineral containing scaffold groups and compared to a native bone sample. Specifically, ^{31}P -based ssNMR experiments were conducted due to their efficiency in differentiating the different forms of calcium phosphate solids including crystalline minerals and amorphous phases [68, 69], their ability to investigate inorganic-organic hybrid materials including bone tissue and our intact mineralised MEW scaffolds

[70, 71] and finally, their ability to directly detect the so-called amorphous surface layer that coats both bone and bone-like HA nanoparticles [37, 38]. One dimensional (1D) ^{31}P single-pulse (SP) MAS ssNMR spectra of pHA and nnHA MEW scaffolds and of iHA powder were first recorded and compared to an intact cortical bone tissue sample from a 2-year-old sheep (Figure 3A). All spectra display a single resonance centred at around 3 ppm which is a signature of the presence of orthophosphate ions (PO_4^{3-}) in hydroxyapatite structure [68]. In contrast, all spectra clearly display different spectral widths (full width at half maximum, FWHM), with values as follows: [bone mineral = 3.3 ± 0.1 ppm], [pHA = 2.4 ± 0.1 ppm], [nnHA = 2.0 ± 0.2 ppm] and [iHA = 0.8 ± 0.1 ppm]. Higher values correspond to a higher distribution of NMR chemical shifts, and hence, a wider distribution of chemical environments. This wider distribution of chemical environments found in bone mineral is due to the high proportion of monohydrogen-orthophosphate ions (HPO_4^{2-}) present in non-apatitic environments [38].

These non-apatitic environments constitute the previously mentioned amorphous surface layer, whose presence can be detected by applying a two-dimensional (2D) $\{^1\text{H}\}^{31}\text{P}$ Heteronuclear Correlation (HetCor) MAS ssNMR experiment (Figure 3B). This experiment gives rise to a ^1H - ^{31}P correlation map in which a correlation peak is signature of atomic-scale spatial proximities among rigid hydrogen-bearing ions/molecules [displayed along the vertical (^1H) F1 dimension] and phosphorus-bearing ions [displayed along the horizontal (^{31}P) F2 dimension]. Here the upper correlation peak (observable at $\delta^1\text{H} = 0.0$ ppm in the ^1H dimension) corresponds to hydroxyl ions (OH^-) ions near PO_4^{3-} ions in the hydroxyapatite's crystal lattice forming the crystalline core of the bone HA nanoparticles. In contrast, the lower correlation peak (observable in the range of $\delta^1\text{H} = 5\text{--}17$ ppm in the ^1H dimension) corresponds to H_2O molecules close to HPO_4^{2-} ions (observable at $\delta^1\text{H} = 5.5$ ppm) along with HPO_4^{2-} ions (observable from $\delta^1\text{H} = 7.0$ to 17.0 ppm) both present in the amorphous layer that coats bone HA nanoparticles. The 2D $\{^1\text{H}\}^{31}\text{P}$ HetCor MAS ssNMR spectra of pHA MEW scaffold and of iHA powder are shown in Figure 3C and Figure 3D respectively. An attempt was made to record a similar 2D spectrum for the nnHA MEW scaffold, but no signal from the HA nanoparticles could be detected due to its low level of mineral compared to the pHA MEW scaffold. Analogous to bone HA nanoparticles, the lower correlation peak due to the presence of non-apatitic environments in the form of an amorphous surface layer is present for the pHA MEW scaffold but absent for iHA powder. This shows that the former sample is composed of bone-like HA nanoparticles since they are coated by an amorphous surface layer. This also suggests that the iHA sample corresponds to stoichiometric hydroxyapatite [$\text{Ca}_5(\text{PO}_4)_3(\text{OH})$] nanoparticles. The present 2D $\{^1\text{H}\}^{31}\text{P}$ HetCor MAS ssNMR spectra also allow for generating the individual ^{31}P NMR signals of both the apatitic core and the amorphous surface layer belonging to the hydroxyapatite nanoparticles of bone mineral and of the pHA MEW

scaffold (Figure 3E-F). As iHA nanoparticles are solely composed of apatitic environments (Figure 3G), the sum of the F2 slices taken at the OH^- ions position in F1 (blue area in Figure 3D) gives rise to a single and narrow resonance centred at around 3 ppm which is signature of the presence of PO_4^{3-} ions in hydroxyapatite structure.

The individual ^{31}P NMR signals were used to estimate the molar ratio of PO_4^{3-} and HPO_4^{2-} ions respectively present in the apatitic crystalline core and the amorphous surface layer of pHA and nnHA groups. A ratio of $80/20 \pm 5\%$ was estimated for the hydroxyapatite nanoparticles associated with the pHA MEW scaffold while a ratio of $50/50 \pm 5\%$ was found for bone HA nanoparticles from an intact, cortical 2-year-old sheep bone tissue sample [6]. While 2D $\{^1\text{H}\}^{31}\text{P}$ HetCor MAS ssNMR spectra could not be successfully obtained for nnHA scaffolds, it can be deduced from other findings that they are also coated by an amorphous surface layer. This is based on the fact that such amorphous surface layer generally forms for nanocrystalline HA samples synthesized by direct precipitation methods at ambient temperature [48, 72, 73]. It can also be seen that the resonance of the ^{31}P SP MAS ssNMR spectrum of the nnHA scaffold ($\text{FWHM} = 2.0 \pm 0.2$ ppm) is largely wider than that of a stoichiometric hydroxyapatite sample such as iHA powder ($\text{FWHM} = 0.8 \pm 0.1$ ppm) (Figure 3A). Based on the above, the hydroxyapatite nanoparticles of the nnHA MEW scaffold, the molar ratio of PO_4^{3-} and HPO_4^{2-} ions was estimated to be $80/20 \pm 10\%$.

Figure 3. **A** Normalized, 1D ^{31}P single-pulse (SP) MAS ssNMR spectra of native bone mineral, pHA scaffolds, nnHA scaffolds and iHA powder. **B-D** Contour plots of 2D $\{^1\text{H}\}^{31}\text{P}$ Heteronuclear Correlation (HetCor) MAS ssNMR spectra of native bone mineral, pHA scaffolds and iHA powder. **E-G** Non-normalized, individual ^{31}P NMR signals of both the apatitic core (blue) and the amorphous surface layer (orange) for each corresponding group **A-C** respectively. Note: Signals were not strong enough to generate 2D contour plots for the nnHA group. The molar ratio of PO_4^{3-} and HPO_4^{2-} ions, respectively present in the apatitic crystalline core and the amorphous surface layer in **H** pHA and **I** nnHA groups. This was estimated by fitting the respective ^{31}P single pulse (SP) MAS ssNMR spectra of pHA and nnHA groups. Experimental spectra were drawn as solid back lines, while their corresponding fits using two different peaks were drawn as red dashed lines. Those two peaks correspond to the apatitic crystalline core (Lorentzian peak, blue) and to the amorphous surface layer (Gaussian peak, orange). Note: The iHA group was not analysed here due to the absence of an amorphous surface layer as revealed in **D**.

4.2.4 *Surface treatment enhances material hydrophilicity*

The wettability of scaffold groups was investigated (Figure 5A) and found to correlate closely with their calcium content (Figure 2H). This is seen in the complete spreading of water on pHA groups, which has the greatest calcium content. The nnHA group also exhibited a significant degree of hydrophilicity, with a mean contact angle of 56.0°. The C and iHA groups were both shown to be hydrophobic in comparison, with contact angles of 117.3° and 117.1° respectively, demonstrating that mineral in the iHA group is not exposed to a sufficient degree to enhance wettability.

4.3 Mechanical properties are enhanced via HA-incorporation into PCL MEW fibres

Incorporation of HA into PCL in the iHA group is seen to increase its stiffness and yield force, while all coating protocols have minimal influence on mechanical behaviour (Figure 4A,B). Stiffness in the linear elastic region between 0.1 - 0.3 N is greatest in iHA scaffolds (Figure 4C), while stiffness in the toe region is not seen to be significantly altered between groups (Figure 4D). Yield force of iHA scaffolds is also significantly enhanced compared to all other groups, with an average fold increase of 1.4.

The nnHA coating was shown to be stable under various loading regimes (Figure S 5). With a 1 N force under shear, some damaged regions with removed coating could be seen, however, most of the coating remained. Similar findings were found for brushing forces, with the coating holding up well and a consistent spread remaining across most of the scaffold surface. A 10 N force under shear was used as a positive control. This removed most of the mineral from the contacting fibres on both aluminium and latex surfaces, as was expected due to the high resulting pressure on a scaffold of diameter 8 mm.

589

590 **Figure 4** Mechanical characterisation of scaffolds via tensile testing. **A** The incorporation of HA into
591 fibres (iHA) can be seen to alter the mechanical properties of PCL micro-fibres. **B** Scaffolds before
592 tensile testing, and after being loaded to approximately 50 % of the yield strain. **C** Significant increases
593 in stiffness seen in iHA, with **D** no change in toe region stiffness. **E** Yield force of iHA scaffolds is also
594 significantly increased. (n=4).
595

596 **4.4 Attachment and proliferation of hMSCs**

597 Cells adhered to scaffolds with an average seeding efficiency of 25 %, with approximately 1,100 –
598 3,400 cells remaining as quantified after 24 h post seeding (Figure 5B). The initial cell seeding efficiency
599 was lowest in the coated scaffold groups; pHA and nnHA, however, this is not significant. This trend
600 was seen to level off by D7 however, where cell number reaches the initial seeding quantity at a
601 number of 10,000 per scaffold. Cells then proliferated 2-fold per week for the remainder of the study,
602 with an average of 24,000 cells at D14 and 41,000 cells at D21. While there were no significant
603 differences between groups at any time-point, a trend of reduced cell number in the pHA scaffold
604 group was seen at D14 and D21. Adherent hMSCs were commonly found branching across adjacent
605 fibres as has been previously demonstrated with this fibre architecture [35]. Interestingly the pHA

coating resulted in a slightly altered cellular morphology with hMSCs adhering to and spreading across the deposited mineral, masking the underlying fibre architecture (Figure 5C).

Figure 5 Characterisation of **A** wettability via water contact angle in all scaffold groups prior to cell seeding. **B** Cell proliferation as quantified by DNA content (n=4). Proliferation in pHA scaffolds can be seen to be attenuated at D14 and D21 compared to other scaffold groups. **C** SEM imaging of cell seeded scaffolds after 24 h culture.

4.5 Surface treatment significantly enhances hMSC osteogenesis

4.5.1 ALP activity

No significant trends were identified in hMSC ALP expression between each scaffold at D14 and D21. Total ALP activity can however be seen to be greater in nnHA and iHA groups at these time-points (Figure 6A). Of note is the 2.2-fold increase in ALP activity in nnHA groups at D21 compared to D14, in contrast with all other groups which remain constant between these time-points. Normalising these results to cell number reveals no further trends in ALP expression (Figure 6B), which remains relatively constant between groups.

Figure 6 Long term culture and osteogenesis of hMSCs. **A** Total ALP activity and **B** ALP activity normalised to DNA at D14 and D21. **C** Collagen production at D21 with **D** total collagen enhanced in the pHA group compared to control and **E** significant increases in pHA compared to all other groups with results normalised to DNA. **F** Calcium content at D21 with contributions from scaffold and cell mineralisation. **G** Total calcium content is significantly enhanced in nnHA and pHA groups. **H** Calcium content normalised to cell number is also enhanced in these groups compared to C and iHA groups. (n=4). & = statistical significance compared to control and iHA groups (& = p<0.05, && = p<0.01, &&& = p<0.001). * = p<0.05.

4.5.2 hMSC Collagen production

Collagen production is shown to be enhanced in coated scaffold groups (Figure 6C). Total collagen quantity is greatest in pHA and nnHA groups at D21, with a 1.7 and 1.5-fold increase respectively compared to control scaffolds (Figure 6D). This effect is further enhanced in the pHA group when

normalising results to cell number (Figure 6D), which is significantly greater than other groups. Collagen content per cell is 3.4-fold greater in the pHA vs control groups, and over 2.5-fold greater than nnHA and iHA groups.

4.5.3 hMSC calcium production

As with previous outputs of osteogenic differentiation, mineral production is seen to be enhanced in the coated scaffold groups (Figure 6F). These results represent both mineralisation due to calcium production by cells, as well as the calcium present due to the treatments prior to cell seeding. Total mineral in pHA groups was 6.7-fold greater than C and iHA groups (Figure 6G). The highest levels of mineralization following 21 days culture was found on the nnHA scaffolds, over 12-fold greater than the calcium content in the C and iHA scaffolds and 1.8-fold greater than the pHA scaffolds. These findings are further enhanced when considering calcium due to cell mineralisation alone, which was calculated by comparing the mineral content between seeded scaffolds and non-cell seeded scaffolds after 21 days culture. The cell contribution to mineral in the pHA group was 59 %, with the remainder the mineral from the scaffold coating. In stark contrast, 92% of the mineral in nnHA groups is contributed by the cells. Considering this cell mineralisation alone, the nnHA group is over 14-fold greater than the control group and 2.9-fold greater than the pHA group. When total calcium is normalised to cell number at D21 (Figure 6H), calcium in pHA and nnHA groups are seen to be significantly greater than others, with an over 10-fold increase in mineral production per cell in the pHA group and over 12-fold increase in the nnHA group when compared to untreated control PCL scaffolds.

4.6 Coating dissolution after long term culture

After 21 days in culture, the pHA coating was found to be largely intact as seen via SEM imaging, with a slightly rougher surface topography (Figure S 6B) compared to at D1 (Figure S 6A). In stark contrast to this, the nnHA coating is seen to have undergone considerable dissolution, with loss of the nano-needle topography and formation of a coating of nano-spheres with diameter of approximately 5 - 20 nm. Consistent fracturing of this coating can also be seen. The iHA group is also considerably altered, with extensive precipitates seen at the fibre surface in contrast to the relatively smooth fibre topography at D1.

4.7 Mineral architecture mediates BMP2 controlled release and further enhances hMSC osteogenesis

In order to investigate the capacity of mineral architectures to bind, stabilise, and release growth factors, scaffolds were loaded with BMP2 and release kinetics and stem/stromal cell osteogenesis investigated. A more sustained early release is seen in the nnHA group (Figure 7A), with significantly

670 lower release vs the pHA group at day 1 and day 3. Mineral treated scaffold groups demonstrate
671 continued BMP2 release throughout the study, with the nnHA group releasing 12.3 % of its BMP2
672 between D7-D14 and 7.3 % between D14-21. Corresponding figures are [7.5 % and 7.5 %] for pHA,
673 [10.4 % and 5.9 %] for iHA and [3.8 % and 2.3 %] for control scaffolds. This can further be seen in Figure
674 7B, illustrating the lowest release from the nnHA group in the first week, and significantly enhanced
675 release in the nnHA group in the second week. This also demonstrates that the majority of BMP2 is
676 released by control scaffolds by day 7 while mineral treatments sustain continuous release throughout
677 the remainder of the study.

678 The controlled release of BMP2 from the MEW scaffolds is seen to enhance cell proliferation in each
679 group. Of particular note is the significant 1.9-fold increase in cell number in the nnHA group at day
680 21 compared to the control nnHA group, in addition to significantly enhanced DNA in BMP2 treated
681 nnHA vs pHA groups, with a fold change of 2.9 (Figure 7C). The effect of BMP2 delivery on the
682 osteogenic differentiation of hMSCs was investigated via ALP activity, collagen and mineral deposition
683 as before. No significant differences in ALP levels were observed between groups (Figure 7D). Collagen
684 content was also investigated, with the greatest increases in BMP2 treated C and iHA scaffolds
685 compared to untreated control scaffolds (Figure 7E). Overall collagen content was between 16 - 20 μg
686 in all groups, with greatest collagen content in the nnHA group after 21 days. Finally, mineralisation is
687 significantly enhanced in BMP2 treated scaffolds vs untreated controls (Figure 7F), however, there
688 were no significant differences within scaffold groups. The greatest changes are seen in the nnHA and
689 iHA groups, with increases of 259 μM and 372 μM respectively (Figure 7F). In summary, the nnHA
690 group exhibits the greatest potential for modification with additional factors, as seen by the greatest
691 level of sustained protein release, significantly enhanced DNA content, and the greatest levels of
692 collagen and mineral content following 21 days in culture.

695 **Figure 7** **A** Cumulative BMP2 release up to D3 and **B** total BMP2 release for each week (n=4). **C** Number of cells, **D** ALP
696 activity, **E** collagen content and **F** calcium content with BMP-2 treatment at D21 compared to data from untreated scaffolds
697 as presented in previous figures. x = statistical significance between scaffold groups within time points via two-way ANOVA
698 and Tukey post test. * = statistical significance via one-way ANOVA and Tukey multiple comparison test within BMP2 group
699 only. + = statistical significance between groups via two-way ANOVA. & = statistical significance vs control and iHA groups
700 with BMP2 treatment. # = statistical significance between Control and BMP2 in the nnHA group via two-way ANOVA and
701 Bonferroni's multiple comparison test.

702 4.8 The nnHA surface treatment can be applied to different architectures and materials

703 Due to the fact that the nnHA coating significantly enhanced hMSC mineralisation, it was investigated
704 if this method could be applied to other materials to increase the range of potential applications for
705 biomedical devices and tissue engineered scaffolds. This has previously been shown with SBF based
706 coatings and has been validated here (Figure S 8A-C). Regarding the nnHA treatment, an identical
707 coating morphology previously seen in MEW PCL scaffolds (Figure S 7A) was also seen with FDM
708 printed scaffolds (using a lower molecular weight PCL) (Figure S 7B) and Ti6Al4V titanium pieces
709 (Figure S 7C). Furthermore, it was investigated if the amount of mineral and thickness of the coating
710 could be adapted if required, and was shown that a greater amount of mineral is deposited on the

surface of MEW fibres (Figure S 9A), FDM fibres (Figure S 9B) and titanium (Figure S 9C) when the number of coating steps is increased from 3 to 12.

5 Discussion

There is a great need for bioinspired synthetic materials to guide bone regeneration in cases of injury or disease. Cell behaviour and function are mediated by microenvironmental biophysical and biochemical cues, with the architecture and chemistry of the surrounding extracellular matrix being fundamental in this regard. Bone at a cellular level consists of arranged fibres with a cross-fibrillar mineral phase made up of plates which in turn are comprised of nano-sized needle shaped crystals [4]. Recapitulating this unique multiscale architecture may represent an innovative approach to develop effective bioinspired materials to promote bone regeneration. The main findings of this study are twofold. Firstly, we utilised a 90° fibrous MEW scaffold architecture, which we have previously optimised for human MSC osteogenesis [35], and built on this foundation by developing biomimetic mineral functionalisation strategies consisting of either intrafibrillar or cross fibrillar configurations. MEW was used here as a study platform due to its ability to fabricate microfibres mimetic of those found in many biological tissues including bone, however, the focus of this study is on the influence of mineral architecture on osteogenesis. Interestingly using a novel coating approach, we were able to grow nano-needle shaped HA (nnHA) crystals on the surface of the MEW fibres which possess a crystalline hydroxyapatite core and amorphous surface layer, closely mimicking the nano-architecture of bone. These bioinspired nnHA topographies significantly enhanced hMSC osteogenesis over traditional SBF plate HA (pHA) coatings or intrafibrillar HA (iHA) scaffolds. Moreover, given the protein stabilising properties of mineral, we demonstrated that nnHA coating facilitated a controlled release of BMP2 from the scaffold which further enhanced mineral deposition by stem/stromal cells. Secondly, we demonstrated that this surface treatment method may be used to coat other commonly used biomedical materials, such as 3D printed FDM scaffolds and Ti6Al4V titanium commonly used in biomedical devices. This study thus outlines a method for fabricating MEW scaffolds with specific fibrous micro-architectures and bone mimetic nano-needle HA extrafibrillar coatings which hold great promise for bone tissue regeneration, and how this surface treatment method can be leveraged to suit a range of potential applications in the larger domain of biomedical engineering. .

The microarchitecture/topography, material chemistry, and mechanical properties of each MEW scaffolds is altered followed CaP modification. The concentrated SBF pHA coating technique resulted in the formation of plates arranged in a spherulitic like coating morphology, consistent with previously published findings using this approach [64, 67, 74] and typical of the plate or “petal-like” morphology of apatite formed via supersaturated solutions [75]. In contrast, the nnHA coating procedure which

we have developed resulted in a vastly different morphology consisting of a fine, extrafibrillar, nano-needle coating which form occasional aggregates, closely mimicking the fundamental mineral units of bone in terms of morphology and composition recently identified [4]. This coating has a mineral grain size of approximately 100 nm, similar to a grain size which has previously been shown to enhance MSC osteogenesis and osteoinduction *in vivo* [44]. It also has the lowest crystallinity of all groups, being most comparable to bone which is formed of poorly crystallised apatite, and the lowest crystallite size of 85 Å being most comparable to the average value of approximately 200 Å (20 nm) in bone [76]. As mineral for XRD analyses was precipitated in solution and isolated via centrifugation (as full scaffold samples could not be analysed), these findings may not be completely indicative of the final mineral components precipitated on to fibres [67]. Therefore, we further characterised full scaffold constructs via NMR. Here, we found that pHA and nnHA coatings most closely resembled the native structure of bone and were found to contain a bone mimetic crystalline hydroxyapatite core surrounded by an amorphous surface layer. Here, the molar ratio of PO_4^{3-} and HPO_4^{2-} ions present in the apatitic crystalline core and amorphous surface layer, broadly analogous to crystallinity, were evaluated. The pHA and nnHA groups were estimated to have a crystalline/amorphous ratio of 80/20, indicating a much more biomimetic structure closer to the value for native bone (50/50). In contrast, the commercially bought nHA in the iHA group was highly crystalline in nature being composed of purely crystalline hydroxyapatite, which is inconsistent with the structure of native bone. The lower crystallinity and more amorphous structure of the nnHA coating is thus beneficial for bone ingrowth, which is aided by the release of calcium and phosphate ions, making it ideal for tissue engineering (TE) applications. In contrast to this, load bearing coated implants typically use high crystallinity calcium phosphates to enhance *in vivo* stability and avoid rapid dissolution of the coating which may result in short term failure [77]. However, applications such as these may benefit from a more bone mimetic apatite coating on top of a stable HA phase, which has been shown to enhance bonding to bone via apatite being created by the human body and bonding to this layer [66]. Our findings indicate that nHA incorporated within the fibres in the iHA group does not drive osteogenesis alone, with the low mineral content of 3.5wt% a limiting factor and difficult to increase appreciably using MEW. Using other approaches, such as FDM [78], or a hybrid MEW-solvent printing approach, can be used to significantly increase the weight percent of additives, with the latter achieving a 33wt% of strontium-substituted bioactive glass in PCL [79]. Approaches such as these which achieve a high core mineral content have many advantages for bone TE applications, and may be further enhanced by our developed nnHA coating. In summary, we have demonstrated that our nnHA coating procedure closely mimics the nano-architecture, composition and crystal structure of native bone, and has great potential for coating various surfaces and structures to achieve a more bone-mimetic topography.

Bone bioinspired architectures can significantly enhance MSC mediated bone formation, as we have demonstrated via long term in-vitro studies. Cell behaviour was investigated on modified scaffolds via proliferation and markers of osteogenic differentiation. Cell number was greatest in the nnHA group after 21 days, with the lowest number of cells in the pHA group. This is consistent with previous work demonstrating a trend of increased proliferation with reduced HA particle size [43]. We have also demonstrated a corresponding increase in ALP activity in the nnHA group, however, this result is not significant. It is known that osteoprogenitor cells initially produce ALP and secrete collagen type I, with further mineralisation occurring on this matrix at later time-points [80, 81]. We have shown previously that the fibrous architecture of scaffolds can have a significant influence on hMSC collagen production [35]. However, our current findings indicate that HA surface topography does not augment collagen production. Further investigation of calcium deposition demonstrated highly significant upregulation, particularly with nnHA coating. The majority of the contribution towards total calcium content is seen to be due to cell-based mineralisation, indicating the significant influence of nano-scale needles in this extrafibrillar coating in driving stem/stromal cell osteogenic differentiation. There are several likely factors for this result, in addition to the influence of the nano-particle size as discussed previously. Greater dissolution of this coating, as demonstrated by SEM imaging after 21 days in culture will yield a greater release of calcium and phosphate ions into the medium which can interact with cells and allow for reprecipitation, enhancing mineralisation [82]. In addition, an inverse relationship between crystallinity and mineralisation has also been shown [83], providing further evidence for the greater cell mineralisation on our nnHA group, which has the lowest crystallinity, and along with the pHA group, has a bone mimetic amorphous surface layer. This therefore suggests that the superior bone formation demonstrated with the nnHA coating is multifactorial, harnessing several mechanisms to maximise regeneration.

Nano-needle structured HA extrafibrillar coatings effectively bind, and slowly deliver stable BMP2, enhancing hMSC proliferation and bone formation. We investigated the protein binding and release capacity of these scaffold modifications via a BMP2 adsorption study. Recent studies have demonstrated that mineral can stabilise bound proteins, preventing conformational changes, maintaining biological activity for weeks during release and that this stabilisation is enhanced within nanostructured coatings [8]. The nnHA group was shown to facilitate the most sustained release of BMP2 over 21 days. The relatively high surface roughness value and large quantity of nano-granular units present in this coating signal towards its high surface area which is favourable for protein binding [8]. This is a likely contributor to the more sustained protein release in this group. Interestingly, there was a significantly enhanced proliferation of cells in this group demonstrating biological activity, while markers of osteogenic differentiation were also marginally increased. A previous study has reported

similar results, where continuous supplementation of media with BMP2 resulted in enhanced proliferation of hMSCs without initiating osteogenic differentiation [84]. However, many studies have also demonstrated the key role of BMP2 in stimulating osteogenesis [85-87]. We believe that due to the high degree of mineralisation indicating late stage differentiation, day 21 may be too late to capture elevated ALP expression, which is an early differentiation marker. Consistent with previous findings in this paper, we also believe that peak collagen levels for our scaffolds are being reached, as seen by the attenuated difference between groups with BMP2 treatment compared to untreated scaffolds. While no significant increases in calcium content were identified, it must be noted that the levels in nnHA scaffolds are already 14-fold greater than control scaffolds in the absence of BMP2 treatment, and the further increases in mineral with BMP2 treatment in nnHA and iHA groups represent a change several fold-times greater than basal calcium levels in untreated control scaffolds, which we have previously shown to represent significantly enhanced mineralisation compared to random scaffolds [35]. In summary, scaffolds with nnHA coating are shown to significantly enhance stem/stromal cell differentiation, with the controlled release of BMP2 loaded scaffolds further contributing to this effect.

6 Conclusions

In conclusion, we utilised MEW technology to create fibrous 3D micro-architectures and further modified these templates with a novel bioinspired extrafibrillar coating of needle shaped nano-sized crystals (nnHA). This osteogenic fibrous structure and bone mimetic mineral architecture significantly enhanced human MSC osteogenesis over more established plate like mineral coatings. Moreover, extrafibrillar coatings of nano-needles facilitated the binding, stabilisation, and controlled release of BMP2 from the material which further enhanced MSC cell proliferation and bone formation. Finally, we have leveraged findings of this study to demonstrate the versatility of this nnHA coating, which may easily be applied to different biomaterials such as Ti6Al4V titanium, additive manufacturing methods, and scaled up to increase mineral content and coating thickness. This study thus outlines a powerful method for modifying constructs and medical devices with a bone mimetic nano-needle HA extrafibrillar coating, which significantly enhances MSC osteogenesis and therapeutic delivery and thus hold great promise for bone tissue regeneration and repair.

7 Data availability and acknowledgements

7.1 Data availability

The authors declare that all data supporting the finding of this study are available within the paper and its Supplementary Information

7.2 Funding

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7.3 Competing interests

The authors declare no conflicts of interest

8 References

1. Yaszemski, M.J., et al., *Evolution of bone transplantation: molecular, cellular and tissue strategies to engineer human bone*. Biomaterials, 1996. **17**(2): p. 175-85.
2. Winkler, T., et al., *A review of biomaterials in bone defect healing, remaining shortcomings and future opportunities for bone tissue engineering: The unsolved challenge*. Bone Joint Res, 2018. **7**(3): p. 232-243.
3. Tzaphlidou, M., *Bone Architecture: Collagen Structure and Calcium/Phosphorus Maps*. Journal of Biological Physics, 2008. **34**(1-2): p. 39-49.
4. Reznikov, N., et al., *Fractal-like hierarchical organization of bone begins at the nanoscale*. Science, 2018. **360**(6388).
5. Von Euw, S., et al., *Organization of Bone Mineral: The Role of Mineral–Water Interactions*. Geosciences, 2018. **8**.
6. Glimcher, M.J., *Bone: Nature of the Calcium Phosphate Crystals and Cellular, Structural, and Physical Chemical Mechanisms in Their Formation*. Reviews in Mineralogy and Geochemistry, 2006. **64**(1): p. 223-282.
7. Asara, J.M., et al., *Protein sequences from mastodon and Tyrannosaurus rex revealed by mass spectrometry*. Science, 2007. **316**(5822): p. 280-5.
8. Yu, X., et al., *Nanostructured Mineral Coatings Stabilize Proteins for Therapeutic Delivery*. Adv Mater, 2017. **29**(33).
9. Alford, A.I., K.M. Kozloff, and K.D. Hankenson, *Extracellular matrix networks in bone remodeling*. The International Journal of Biochemistry & Cell Biology, 2015. **65**: p. 20-31.
10. Rutkovskiy, A., K.-O. Stensløkken, and I.J. Vaage, *Osteoblast Differentiation at a Glance*. Medical Science Monitor Basic Research, 2016. **22**: p. 95-106.
11. Nair, A.K., et al., *Molecular mechanics of mineralized collagen fibrils in bone*. Nature Communications, 2013. **4**(1): p. 1724.
12. Crockett, J.C., et al., *Bone remodelling at a glance*. Journal of Cell Science, 2011. **124**(7): p. 991.

13. Kane, R. and P.X. Ma, *Mimicking the nanostructure of bone matrix to regenerate bone*. *Materials Today*, 2013. **16**(11): p. 418-423.
14. Weiner, S. and H.D. Wagner, *THE MATERIAL BONE: Structure-Mechanical Function Relations*. *Annual Review of Materials Science*, 1998. **28**(1): p. 271-298.
15. Lu, P., et al., *Extracellular Matrix Degradation and Remodeling in Development and Disease*. *Cold Spring Harbor perspectives in biology*, 2011. **3**(12): p. 10.1101/cshperspect.a005058 a005058.
16. Bonnans, C., J. Chou, and Z. Werb, *Remodelling the extracellular matrix in development and disease*. *Nature reviews. Molecular cell biology*, 2014. **15**(12): p. 786-801.
17. Hutmacher, D.W., et al., *Mechanical properties and cell cultural response of polycaprolactone scaffolds designed and fabricated via fused deposition modeling*. *J Biomed Mater Res*, 2001. **55**(2): p. 203-16.
18. Di Luca, A., et al., *Tuning Cell Differentiation into a 3D Scaffold Presenting a Pore Shape Gradient for Osteochondral Regeneration*. *Advanced Healthcare Materials*, 2016. **5**(14): p. 1753-1763.
19. Daly, A.C., et al., *3D Bioprinting for Cartilage and Osteochondral Tissue Engineering*. *Advanced Healthcare Materials*, 2017. **6**(22): p. 1700298.
20. Cox, S.C., et al., *3D printing of porous hydroxyapatite scaffolds intended for use in bone tissue engineering applications*. *Materials Science and Engineering: C*, 2015. **47**: p. 237-247.
21. Fierz, F.C., et al., *The morphology of anisotropic 3D-printed hydroxyapatite scaffolds*. *Biomaterials*, 2008. **29**(28): p. 3799-3806.
22. Bergmann, C., et al., *3D printing of bone substitute implants using calcium phosphate and bioactive glasses*. *Journal of the European Ceramic Society*, 2010. **30**(12): p. 2563-2567.
23. Inzana, J.A., et al., *3D printing of composite calcium phosphate and collagen scaffolds for bone regeneration*. *Biomaterials*, 2014. **35**(13): p. 4026-4034.
24. Hoglebe, N.J., J.W. Reinhardt, and K.J. Gooch, *Biomaterial microarchitecture: a potent regulator of individual cell behavior and multicellular organization*. *Journal of Biomedical Materials Research Part A*, 2017. **105**(2): p. 640-661.
25. Labour, M.-N., et al., *Electrospun Poly-D-L-Lactic Acid Fibrous Scaffolds as a Delivery Vehicle for Calcium Phosphate Salts to Promote In Situ Mineralisation and Bone Regeneration*. *Journal of Biomaterials and Tissue Engineering*, 2018. **8**(2): p. 206-217.
26. Zhou, P., et al., *Loading BMP-2 on nanostructured hydroxyapatite microspheres for rapid bone regeneration*. *International Journal of Nanomedicine*, 2018. **13**: p. 4083-4092.
27. Brown, T.D., P.D. Dalton, and D.W. Hutmacher, *Direct writing by way of melt electrospinning*. *Adv Mater*, 2011. **23**(47): p. 5651-7.
28. Hochleitner, G., et al., *Additive manufacturing of scaffolds with sub-micron filaments via melt electrospinning writing*. *Biofabrication*, 2015. **7**(3).
29. Wunner, F.M., et al., *Melt Electrospinning Writing of Three-dimensional Poly(epsilon-caprolactone) Scaffolds with Controllable Morphologies for Tissue Engineering Applications*. *J Vis Exp*, 2017(130).
30. de Ruijter, M., et al., *Out-of-Plane 3D-Printed Microfibers Improve the Shear Properties of Hydrogel Composites*. *Small*, 2017: p. 1702773-n/a.
31. Castilho, M., et al., *Melt Electrowriting Allows Tailored Microstructural and Mechanical Design of Scaffolds to Advance Functional Human Myocardial Tissue Formation*. *Advanced Functional Materials*, 2018. **0**(0): p. 1803151.
32. Hochleitner, G., et al., *Melt electrowriting below the critical translation speed to fabricate crimped elastomer scaffolds with non-linear extension behaviour mimicking that of ligaments and tendons*. *Acta Biomaterialia*, 2018. **72**: p. 110-120.
33. Brennan, C.M., K.F. Eichholz, and D.A. Hoey, *The effect of pore size within fibrous scaffolds fabricated using melt electrowriting on human bone marrow stem cell osteogenesis*. *Biomedical Materials*, 2019. **14**(6): p. 065016.

34. Tylek, T., et al., *Precisely defined fiber scaffolds with 40 μ m porosity induce elongation driven M2-like polarization of human macrophages*. Biofabrication, 2020. **12**(2): p. 025007.
35. Eichholz, K.F. and D.A. Hoey, *Mediating human stem cell behaviour via defined fibrous architectures by melt electrospinning writing*. Acta Biomater, 2018. **75**: p. 140-151.
36. Rey, C., et al., *Bone mineral: update on chemical composition and structure*. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA, 2009. **20**(6): p. 1013-1021.
37. Von Euw, S., et al., *Amorphous surface layer versus transient amorphous precursor phase in bone - A case study investigated by solid-state NMR spectroscopy*. Acta Biomater, 2017. **59**: p. 351-360.
38. Von Euw, S., et al., *Bone mineral: new insights into its chemical composition*. Sci Rep, 2019. **9**(1): p. 8456.
39. Boskey, A.L., *Bone composition: relationship to bone fragility and antiosteoporotic drug effects*. BoneKEy Reports, 2013. **2**: p. 447.
40. Habraken, W., et al., *Calcium phosphates in biomedical applications: materials for the future?* Materials Today, 2016. **19**(2): p. 69-87.
41. Lc, C., *Next generation calcium phosphate-based biomaterials*. Dental materials journal, 2009. **28**(1): p. 1-10.
42. Zhou, H. and J. Lee, *Nanoscale hydroxyapatite particles for bone tissue engineering*. Acta Biomaterialia, 2011. **7**(7): p. 2769-2781.
43. Cai, Y., et al., *Role of hydroxyapatite nanoparticle size in bone cell proliferation*. Journal of Materials Chemistry, 2007. **17**(36): p. 3780-3787.
44. Li, X., et al., *Design of hydroxyapatite bioceramics with micro-/nano-topographies to regulate the osteogenic activities of bone morphogenetic protein-2 and bone marrow stromal cells*. Nanoscale, 2020. **12**(13): p. 7284-7300.
45. Yang, X., et al., *In Vitro Uptake of Hydroxyapatite Nanoparticles and Their Effect on Osteogenic Differentiation of Human Mesenchymal Stem Cells*. Stem cells international, 2018. **2018**: p. 2036176-2036176.
46. Remya, N.S., et al., *An in vitro study on the interaction of hydroxyapatite nanoparticles and bone marrow mesenchymal stem cells for assessing the toxicological behaviour*. Colloids and Surfaces B: Biointerfaces, 2014. **117**: p. 389-397.
47. Liu, Y., et al., *In vitro effects of nanophase hydroxyapatite particles on proliferation and osteogenic differentiation of bone marrow-derived mesenchymal stem cells*. Journal of Biomedical Materials Research Part A, 2008. **90A**(4): p. 1083-1091.
48. Mahon, O.R., et al., *Nano-particle mediated M2 macrophage polarization enhances bone formation and MSC osteogenesis in an IL-10 dependent manner*. Biomaterials, 2020. **239**: p. 119833.
49. Domingos, M., et al., *Three-dimensional printed bone scaffolds: The role of nano/micro-hydroxyapatite particles on the adhesion and differentiation of human mesenchymal stem cells*. Proc Inst Mech Eng H, 2016: p. 954411916680236.
50. Li, B., et al., *Preparation of nano-hydroxyapatite particles with different morphology and their response to highly malignant melanoma cells in vitro*. Applied Surface Science, 2008. **255**(2): p. 357-360.
51. Duan, R., et al., *Accelerated bone formation by biphasic calcium phosphate with a novel sub-micron surface topography*. Eur Cell Mater, 2019. **37**: p. 60-73.
52. Xiong, L., et al., *BMP2-loaded hollow hydroxyapatite microspheres exhibit enhanced osteoinduction and osteogenicity in large bone defects*. International journal of nanomedicine, 2015. **10**: p. 517-526.

53. Dellinger, J.G., J.A.C. Eurell, and R.D. Jamison, *Bone response to 3D periodic hydroxyapatite scaffolds with and without tailored microporosity to deliver bone morphogenetic protein 2*. Journal of Biomedical Materials Research Part A, 2005. **76A**(2): p. 366-376.
54. Notodihardjo, F.Z., et al., *Bone regeneration with BMP-2 and hydroxyapatite in critical-size calvarial defects in rats*. Journal of Cranio-Maxillofacial Surgery, 2012. **40**(3): p. 287-291.
55. Gonzalez-Fernandez, T., et al., *Mesenchymal stem cell fate following non-viral gene transfection strongly depends on the choice of delivery vector*. Acta Biomaterialia, 2017. **55**: p. 226-238.
56. Curtin, C.M., et al., *Innovative Collagen Nano-Hydroxyapatite Scaffolds Offer a Highly Efficient Non-Viral Gene Delivery Platform for Stem Cell-Mediated Bone Formation*. Advanced Materials, 2012. **24**(6): p. 749-754.
57. Palazzo, B., et al., *Biomimetic Hydroxyapatite-Drug Nanocrystals as Potential Bone Substitutes with Antitumor Drug Delivery Properties*. Advanced Functional Materials, 2007. **17**(13): p. 2180-2188.
58. Sun, W., et al., *Biodegradable Drug-Loaded Hydroxyapatite Nanotherapeutic Agent for Targeted Drug Release in Tumors*. ACS Applied Materials & Interfaces, 2018. **10**(9): p. 7832-7840.
59. Martine, L.C., et al., *Engineering a humanized bone organ model in mice to study bone metastases*. Nat. Protocols, 2017. **12**(4): p. 639-663.
60. Kumta, P.N., et al., *Nanostructured calcium phosphates for biomedical applications: novel synthesis and characterization*. Acta Biomaterialia, 2005. **1**(1): p. 65-83.
61. Cunniffe, G.M., et al., *The synthesis and characterization of nanophase hydroxyapatite using a novel dispersant-aided precipitation method*. J Biomed Mater Res A, 2010. **95**(4): p. 1142-9.
62. Stavenschi, E., et al., *Physiological cyclic hydrostatic pressure induces osteogenic lineage commitment of human bone marrow stem cells: a systematic study*. Stem Cell Research & Therapy, 2018. **9**(1): p. 276.
63. Kokubo, T., et al., *Ca,P-rich layer formed on high-strength bioactive glass-ceramic A-W*. J Biomed Mater Res, 1990. **24**(3): p. 331-43.
64. Kokubo, T., et al., *Solutions able to reproduce in vivo surface-structure changes in bioactive glass-ceramic A-W3*. Journal of Biomedical Materials Research, 1990. **24**(6): p. 721-734.
65. Oyane, A., et al., *Simple surface modification of poly(ϵ -caprolactone) to induce its apatite-forming ability*. Journal of Biomedical Materials Research Part A, 2005. **75A**(1): p. 138-145.
66. Kokubo, T. and H. Takadama, *How useful is SBF in predicting in vivo bone bioactivity?* Biomaterials, 2006. **27**(15): p. 2907-15.
67. Vaquette, C., et al., *Effect of culture conditions and calcium phosphate coating on ectopic bone formation*. Biomaterials, 2013. **34**(22): p. 5538-51.
68. Rothwell, W.P., J.S. Waugh, and J.P. Yesinowski, *High-resolution variable-temperature phosphorus-31 NMR of solid calcium phosphates*. Journal of the American Chemical Society, 1980. **102**(8): p. 2637-2643.
69. Pourpoint, F., et al., *Calcium Phosphates and Hydroxyapatite: Solid-State NMR Experiments and First-Principles Calculations*. Applied Magnetic Resonance, 2007. **32**(4): p. 435-457.
70. Santos, R.A., R.A. Wind, and C.E. Bronnimann, *^1H CRAMPS and ^1H - ^{31}P HetCor experiments on bone, bone mineral, and model calcium phosphate phases*. J Magn Reson B, 1994. **105**(2): p. 183-7.
71. Wang, Y., et al., *Water-mediated structuring of bone apatite*. Nat Mater, 2013. **12**(12): p. 1144-53.
72. Jaeger, C., et al., *A Solid-State NMR Investigation of the Structure of Nanocrystalline Hydroxyapatite*. Magnetic resonance in chemistry : MRC, 2006. **44**: p. 573-80.
73. Wang, Y., et al., *Impact of collagen confinement vs. ionic substitutions on the local disorder in bone and biomimetic apatites - SI*. 2016.

74. Tas, A.C. and S.B. Bhaduri, *Rapid coating of Ti6Al4V at room temperature with a calcium phosphate solution similar to 10× simulated body fluid*. Journal of Materials Research, 2011. **19**(9): p. 2742-2749.
75. Drouet, C., *Apatite Formation: Why It May Not Work as Planned, and How to Conclusively Identify Apatite Compounds*. BioMed Research International, 2013. **2013**: p. 490946.
76. Danilchenko, S., et al., *Determination of the Bone Mineral Crystallite Size and Lattice Strain from Diffraction Line Broadening*. Crystal Research and Technology, 2002. **37**: p. 1234-1240.
77. Levingstone, T.J., et al., *Plasma sprayed hydroxyapatite coatings: Understanding process relationships using design of experiment analysis*. Surface and Coatings Technology, 2015. **283**: p. 29-36.
78. Diloksumpan, P., et al., *Combining multi-scale 3D printing technologies to engineer reinforced hydrogel-ceramic interfaces*. Biofabrication, 2020. **12**(2): p. 025014.
79. Paxton, N.C., et al., *Rheological Characterization of Biomaterials Directs Additive Manufacturing of Strontium-Substituted Bioactive Glass/Polycaprolactone Microfibers*. Macromolecular Rapid Communications, 2019. **40**(11): p. 1900019.
80. Huang, Z., et al., *The sequential expression profiles of growth factors from osteoprogenitors [correction of osteoprogenitors] to osteoblasts in vitro*. Tissue Eng, 2007. **13**(9): p. 2311-20.
81. Birmingham, E., et al., *Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche*. Eur Cell Mater, 2012. **23**: p. 13-27.
82. Chang, Y.-L., C.M. Stanford, and J.C. Keller, *Calcium and phosphate supplementation promotes bone cell mineralization: Implications for hydroxyapatite (HA)-enhanced bone formation*. Journal of Biomedical Materials Research, 2000. **52**(2): p. 270-278.
83. Morgan, J., et al., *In vitro mineralization and implant calcium phosphate-hydroxyapatite crystallinity*. Implant Dent, 1996. **5**(4): p. 264-71.
84. Lysdahl, H., et al., *Preconditioning Human Mesenchymal Stem Cells with a Low Concentration of BMP2 Stimulates Proliferation and Osteogenic Differentiation In Vitro*. BioResearch Open Access, 2014. **3**(6): p. 278-285.
85. Beederman, M., et al., *BMP signaling in mesenchymal stem cell differentiation and bone formation*. Journal of biomedical science and engineering, 2013. **6**(8A): p. 32-52.
86. Huang, W., et al., *BMP-2 exerts differential effects on differentiation of rabbit bone marrow stromal cells grown in two-dimensional and three-dimensional systems and is required for in vitro bone formation in a PLGA scaffold*. Experimental Cell Research, 2004. **299**(2): p. 325-334.
87. Li, C., et al., *Electrospun silk-BMP-2 scaffolds for bone tissue engineering*. Biomaterials, 2006. **27**(16): p. 3115-3124.

9 Supplementary tables and figures

Table S 1 Fibre diameter for all groups and resulting coating thickness of pHA and nHA groups as calculated via the difference between these and the control group

units: μm	Control		pHA		nnHA		iHA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Fibre diameter	9.09	1.00	13.53	2.63	10.19	1.02	11.79	4.19
Coating thickness	-		2.22		0.55		-

Figure S 1 Distribution of nHA within MEW fibres for **A** an early print with the initial material in the syringe and **B** a late print with the last of the material in the syringe. **C** Thermogravimetric analysis (TGA) verifying the quantity of mineral midway through the print.

Figure S 2 Element mapping of SEM images via EDX analysis illustrating the distribution of calcium and phosphorus and representative EDX spectra for all groups.

Figure S 3 Surface topography and roughness (R_a , units = μm) determined using white light interferometry for **A** uncoated control samples **B** PHA and **C** nnHA groups.

Figure S 4 XRD analysis of powder representative of CaP, nnHA and iHA groups.

Figure S 5 Stability of nnHA coating. A shear force of 1 N or 10 N was applied to scaffolds on both aluminium and latex surfaces to assess stability of the coating. Stability was also investigated under brushing.

Figure S 6 Fibre appearance after **A** day 1 and **B** day 21 in culture. Plates in the pHA group appear more rough at D21, with morphology otherwise unchanged. The nnHA coating is significantly altered, with the initial nano-needles becoming spherical and smaller in nature, and the coating exhibiting widespread fracturing. A greater amount of mineral precipitation on the fibre surface is seen in iHA at D21, where the near-surface HA appears to act as sites for mineral nucleation.

Figure S 7 Versatility of nnHA coating. In addition to **A** MEW, the nnHA coating was successfully applied to both **B** FDM scaffolds and **C** Ti-6Al-4V titanium surfaces using the same protocol and with resulting identical HA nanotopography.

Figure S 8 Validation of the ability to coat different scales and materials with pHA using **A** MEW with 80 kDa PCL, **B** FDM with 50 kDa PCL and **C** Ti6Al4V.

Figure S 9 Coating thickness and total mineral content can easily be adapted. Illustration of the effect of number of the nnHA coatings when treating different surfaces including **A** MEW with 80 kDa PCL, **B** FDM with 50 kDa PCL and **C** Ti6Al4V.