Osteoinduction of particulate and shot-fibre reinforced composites using PLLA and Apatite-Wollastonite

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Abstract: Composites have clinical application for their ability to mimic the hierarchical structure of human tissues. The combination of materials with desired properties is a key advantage, particularly for tissue engineering applications where both tissue support and cellular stimulation are needed. The use of degradable matrices reinforced by bioactive ceramics is seen as a viable process to increase osteoconductivity and accelerate tissue regeneration. Technologies such as additive manufacturing, provide the design freedom needed to create patient-specific implants with complex shapes and controlled porous structures. In this study a medical grade poly(L-lactide) (PLLA) was used as matrix while apatite-wollastonite (AW) was used as reinforcement (5 wt% loading). Premade rods of composite were pelletized and processed to create a filament with an average diameter of 1.6 mm, using a twin screw extruder. The resultant filament was 3D printed into 3 types of porous woodpile samples, PLLA, PLLA reinforced with AW particles and PLLA with AW fibres. Mechanical testing showed that the fibre reinforced composite has higher flexural modulus. Degradation studies indicated that all samples' integrity was maintained after immersion in phosphate buffered solution for 8 weeks. An average effective modulus of 0.8 GPa, 1 GPa and 1.5GPa was obtained for the polymer, particle and fibre composite, respectively. Composite samples immersed in simulated body fluid exhibited bioactivity, producing a surface apatite layer. Furthermore, cell viability and differentiation were

demonstrated in human mesenchymal stromal cells for all sample types, with mineralisation detected solely for biocomposites. It is concluded that both composites have potential for use in critical size bone defects, with the AW fibre composite showing greater levels of ion release, stimulating more rapid cell proliferation and greater levels of mineralisation during cells assays.

Keywords: biocomposite, bioactive, osteoinduction, mineralisation.

1. Introduction

A rising number of bone defect-related pathologies, such as osteoporosis and osteoarthritis, are registered every year. The current gold standards for treatment are bone fillers and grafts but their use is limited by size and sourcing difficulties [1,2]. Tissue engineering (TE) approaches, using scaffolds as tailored platforms, aim to promote and support tissue growth to repair defects[3,4].

Bone contains both a mineral phase and an organic phase, and can be considered a natural composite where bone apatite is reinforced with collagen fibres[5,6]. Composite materials for implants have been investigated for their versatility, seeking a balance between the mechanical and biological properties of the constituent materials. A common model for bone scaffolds is polymer materials with particulate bioceramic reinforcement, commonly Bioglass®, hydroxyapatite (HA) and tri-calcium phosphate (TCP). The incorporation of these fillers into matrices such as poly (l-lactic acid) (PLLA), poly (lactic-*co*-glycolic acid) (PLGA) and high density polyethylene (HDPE), have demonstrated improved mechanical properties, osteoconductivity and enhanced expression of mature bone marker genes such as osteocalcin [5,7–10]. Improved version of bioglasses were created to enable use in load bearing areas and to improve osteointegration. Kokubo et al., created the apatite-wollastonite glass-ceramic (AW), by modifying the composition and processing of bioactive glasses. This allowed the precipitation of Oxyfluorapatite and Wollastonite crystals, providing AW with a superior

mechanical strength, toughness and stability in physiological environment. Un-sintered, AW has an appropriate composition for bioactivity ($\text{SiO}_2 - \text{CaO} - \text{MgO} - \text{P}_2\text{O}_5 - \text{F}$), with the higher content of CaO contributing to a high leaching of Ca^{2+} , that dissolves into the surrounding body fluid and bonds with the Si-OH on the surface of the implant to form the apatite nucleation site[11].

Polymer-ceramic composites can be produced via two routes: physicochemical and thermo-mechanical, the latter being ideal for industrial adaptation but with design limitations[12]. Additive manufacturing (AM), has an ability to generate complex shapes with high porosity, at various scales[13]. Fused Filament Fabrication (FFF) is one of the most cost-effective technologies in AM, therefore appealing for small-scale production. Most bioactive composite printing has been performed on particulate reinforced systems, mainly using calcium phosphates. Short fibre reinforced systems have not been extensively investigated, but offer the potential for greater presentation of the ceramic at the surface of the composite (reference previous paper).

In this study we explore FFF as a processing technique for bioactive composite scaffolds, using PLLA as matrix and AW precursor glass in particle and short fibre form, with the main aim to assess the influence of both AW fillers on mechanical properties, degradation profile and impact on human mesenchymal stromal cell (hMSC) differentiation and mineralisation, compared with net polymer constructs.

2. Materials and Methods

Un-sintered AW precursor glass was provided by Glass Technology Services (Sheffield, United Kingdom), with the following composition (in wt %) 4.6MgO-44.7CaO-34SiO₂-16.2P₂O₅- 0.5CaF₂ [14], and density of 3.087g/cm³[15]. The average powder particle size was 14 µm (with a standard deviation of 1µm) and the average fibre length was 60 µm (with a

standard deviation of 9 μ m). As matrix, medical graded PLLA (Purasorb PL 38, Corbion - Purac Biomaterials) with an average density is 1.24g/cm³, was used.

2.1 Production of the composite samples

2.1.1 Material blending and filament fabrication

The incorporation of ceramic into the polymer matrix involved mixing 1.90 $\pm$ 0.02g of PLLA granules and 0.10 $\pm$ 0.02g of AW (particle or short-fibre) in an aluminium mould to form rods measuring 200 x 3 x 4mm. The rods were then pelletized to create granules that were fed into a twin screw extruder (Rondol Technology Ltd, United Kingdom) to produce a filament. For the extrusion process, a maximum temperature of 190°C was applied, which then reduced to 160°C at the die. The extruded material was inserted in a haul-off machine set to move at 0.7mm/s. The resultant filament diameter was 1.6 mm (with a standard deviation of 0.2 mm).

2.1.2 Sample preparation: Fused Filament Fabrication

A CAD file for woodpile scaffold design was prepared in Autodesk Inventor (©Autodesk Inc.) and exported into the printer's slicing software (CraftWare, CraftUnique Ltd). The filament prepared in 2.1.1 was fed into the printer (Craftbot 2, CraftUnique, Hungary) with a nozzle heated to 230°C. The molten filament was selectively deposited onto a building platform, heated at 60°C, on a layer by layer fashion. The printing parameters were defined as 0.7 $\pm$ 0.1 mm extrusion width, 0.2 mm layer height and 30 mm/s of draw speed. Each part was printed individually to avoid failure from filament discrepancies and no post processing or supports were needed. Two types of samples were designed (figure sup 1), squared scaffolds (10 x 10 x 2 mm) for structural and chemical characterisation, and for the biological assay, and bars (10 x 40 x 3 mm) for 3-point bend test, before and after degradation.

2.2 Material characterisation

2.2.1 Mechanical properties

The mechanical properties (n=5 per material) were assessed through 3-point bend test (span length of 32 mm), using 1 mm/ min cross-head speed on a standard testing machine (Shimadzu, United Kingdom).

2.2.2 X-Ray Diffraction (XRD)

The crystallinity of raw material and printed parts was measured with a PANalytical X'Pert Pro MPD, powered by a Philips PW3040/60 X-ray generator. The machine was fitted with an X'Celerator detector and the diffraction data obtained by exposing the samples to Cu-K α X-ray radiation. Radiation was characterised by a wavelength (λ) of 1.5418 Å and the X-rays generated from a Cu anode supplied with 40 kV and a current of 40 mA.

2.2.3 Diffusion Ordered Nuclear Magnetic Resonance Spectroscopy (DOSY)

Diffusion NMR ^1H proton measurements were used to determine polymer chains hydrodynamic radii. These were carried out using a Bruker Avance III 400 MHz nuclear magnetic resonance (NMR) spectrometer, equipped with a 5 mm SMART probe. All measurements were carried out at 298 K using Bruker ICON automation software and processing carried out using Bruker Topspin software (V3.6.1). Samples were analysed using a bipolar gradient pulse (the pulse sequence is described in the supplementary information). Samples were dissolved in deuterated chloroform (CDCl_3) (1 mg ml^{-1} concentration) and analysed across 64 magnetic gradient intervals to determine polymer and solvent diffusion[16]. The relative sample viscosity of the sample was determined via comparison to a CDCl_3 blank ($D = 2.59 \times 10^{-9} \text{ m}^2 \text{ S}^{-1}$) with a known solvent viscosity ($5.28 \times 10^{-4} \text{ kg m}^{-1} \text{ s}^{-1}$)[17]. Sample viscosity was determined via changes in solvent diffusion and thus a corrected polymer material hydrodynamic was determined from PLLA ^1H proton diffusional shift.

2.3 *In vitro* degradation assay

2.3.1 Mass loss, pH variations and ion leaching

Samples were weighted (n=3 per material) using an analytical balance (Sartorius, Sartorius AG), then placed into individual plastic vials containing Phosphate Buffered Solution (PBS) (Sigma Aldrich, UK) with average pH 7.4 and incubated at 37°C. The amount of PBS for square and bar shaped samples was 5 ml and 25 ml, respectively. Measurements were taken at week 1, 2, 4, 6 and 8 for pH, flexural strength and modulus, and mass. The solution was refreshed weekly and the pH of the solution measured using a portable FG2-Kit Five Go™ pH meter (Mettler Toledo Ltd, UK). To assess the mass loss, samples were incubated at 37°C for 48 h, and re-weighted to obtain the dry weight. Flexural strength was evaluated according to the method described on par. 2.1.1.

The collected PBS solution was analysed by inductively coupled plasma mass spectrometry (ICP-MS) on a Thermo-Fisher X-series2 instrument operating in collision cell mode (using 3 mL min⁻¹ 8% H₂ in He as collision gas) for elemental detection. The samples were diluted 5-fold in 2.5% (w/v) high-purity HNO₃ (Merck) containing 20 µg/L indium (In) and platinum (Pt) as internal elemental standards. For each sample, magnesium (²⁴Mg), calcium (⁴⁰Ca), indium (¹¹⁵In), and platinum (¹⁹⁵Pt) isotopes were monitored sequentially using the peak-jump method (100 individual reads of 30 ms on each isotope, across 3 channels of 0.02 atomic mass unit separation, each analysed in technical triplicate). Elemental concentrations were determined by comparison with standard solutions (Merck) of known elemental composition (0 – 1,000 µg/L) analysed within the same run.

2.4 Bioactivity assay – immersion in simulated body fluid (SBF)

The samples were immersed in 5ml SBF (n=3 per material) and measurements were taken at day 1, 7, 14 and 21. SBF was prepared according to the instructions provided by Kokubo et al., 2006[18], with a final ionic concentration (in mM) of 142Na⁺, 5K⁺, 1.5Mg²⁺, 2.5Ca²⁺, 147.8Cl⁻, 4.2HCO₃⁻, 1HPO₄²⁻ and SO₄²⁻ (in mM), with pH 7.42, at 36.5°C. The samples were incubated at 37°C and the solution refreshed weekly. After the pH was recorded, samples

were gently immersed in di-ionised water for 30s to removed precipitated salt and then dried in the incubator at 37°C for 48h for microscope analysis.

2.5 Cell studies on composites scaffolds

2.5.1 Cell seeding method

An immortalized cell line[19] of TERT Human Mesenchymal Stromal Cells (hTERT-MSCs) Y201 was used as an *in vitro* model, at passage 87. Sterilisation of scaffolds was performed in two steps, starting with immersion in 70% ethyl alcohol solution (Sigma Aldrich, UK) for 15 minutes. Scaffolds were then exposed to UV irradiation, on a 302 nm waveband (EL Series UV Lamps, Ultra-Violet Products Ltd.), for 40 minutes. Sterilisation was followed by sample washing, using a sterile Dulbecco's PBS (DPBS; Sigma Aldrich, UK). Washed sample were placed into 12 well plates, and hTERT MSCs were seeded in triplicate on top of them, at a density of 50 000 cells/scaffold. Incubation was done at 37°C, in a humidified atmosphere of 5% v/v CO₂, and the culture period defined as 21 days. Cells were maintained in Dulbecco's modified eagle medium, (DMEM) with high glucose, sodium pyruvate, L-glutamine and phenol red (Gibco; Thermo Fisher Scientific). The media was supplemented with 10% Fetal Bovine Serum (Gibco; Thermo Fisher Scientific) and 1% Penicillin-Streptomycin (Sigma Aldrich, UK) and refreshed every 2 days. Tissue culture plastic (TCP) was chosen for the positive control.

2.5.2 MTT (3-dimethylthiazol-2,5-diphenyltetrazolium bromide) colorimetric assay

The metabolic activity of cells was determined by MTT (Thiazolyl Blue Tetrazolium Bromide) using a standard kit provided by Sigma Aldrich (UK). The MTT solution was made following the supplier instructions, attaining a final concentration of 5 mg/mL, in DPBS. The stock solution was mixed with serum-free DMEM deprived of phenol red (Gibco; Thermo Fisher, UK). Measurements were taken in triplicate after 1, 3, 7, 14 and 21 days. Samples were incubated for 4 hours at room temperature with protection from light. The MTT media

was removed, 400 μ L isopropanol were added and the plates manually agitated for 30 minutes. This procedure aimed the dissolution of formazan crystals, a product of digestion of the MTT by the cells. For the absorbance reading, 200 μ L of the dissolved formazan were pipetted into a 96-well plate, which was placed on spectrometer (ELx800; BioTek Instruments, UK), at 570 nm. The estimation of the cell number was based a standard curve, generated by seeding hTERT-MSCs in different densities (0, 10 000, 30 000, and from there on up to 450 000 with a 50 000 increase).

2.5.3 Live and dead staining

Cytotoxicity was assessed via a live/dead kit (Thermo Fisher, UK), for day 1 and day 3. The stock solutions were prepared according to the manufacturer's instructions, leading to a final concentration of 4 μ M ethidium homodimer-1 (EthD1) and 2 μ M calcein. First, the culture media was removed from the wells and the scaffolds washed twice with PBS. The created solutions were pipetted into the wells, and the plates incubated for 30 minutes, at room temperature and protected from light. The observation was performed in an inverted confocal microscope (LSM 800; Carl Zeiss Ltd). For this, each sample was placed in a supplied plastic dish (seeding surface facing the lens), and DPBS added to avoid sample drying. Predefined laser wavelengths were applied, AF488 (410-546nm) for green (live) and AF546 (576-700nm) for red (dead), and images obtained using the supplier's software (ZEN lite, Zeiss).

2.5.4 Cell fixation and staining for confocal microscopy

Morphological observation of cells was performed by confocal microscopy, for samples collected at day 1, 7 and 21. The process started with aspiration of cellular media and sample washing with DPBS. For the fixation, a solution of 4% paraformaldehyde (Sigma Aldrich) was added to the wells and the plates incubated for 15 minutes, at room temperature. Prior to staining, cells were washed 3 times with a DPBS/0.1% Tween 20 solution. The staining began by immersing the cells in 3% w/v goat's serum/DPBS/0.1% Tween 20, and incubation for 30

minutes at room temperature. Next, the primary antibody solution was prepared, added to the wells, and incubated for 1 hours at room temperature (protected from light). The solution consisted of 3%w/v Goat's serum/DPBS/0.1%Tween 20 and Vinculin (ABfinity rabbit; Thermo Fisher), on a ratio of 1:200. Additional washing with DPBS/0.1%Tween 20 preceded the immersion in the secondary antibody (Alexa Fluor 488, donkey anti-mouse antibody; Life Technologies), formulated and incubated under the same conditions defined for the primary antibody. The secondary antibody was washed a solution containing phalloidin-tetramethylrhodamine B isothiocyanate peptide (Sigma Aldrich) and DPBS/0.1%Tween 20 (1:1000 ratio) was applied for 30 minutes, at room temperature and light protected. Another washing was performed, and 4',6-diamidino-2-phenylindole (DAPI; Sigma Aldrich) solution was added, and incubated for 1 hour at room temperature, light-protected. As a final point, samples were washed with DPBS/0.1%Tween 20 once, followed by DPBS wash step.

A second set of samples was stained to detect collagen type 1, the actin filaments and the nucleus. After fixation and washes as in the previous staining, samples immersed in 2 % v/v bovine serum albumine/DPBS/0.1%Tween 20 and incubated for 30 minutes at 4 °C. A primary antibody solution was added, consisting of DPBS/0.1%Tween 20/anti-collagen 1 antibody (ab34710, Abcam plc) on a ratio of 1:1000. Samples were then incubated for 3 hours at 4 °C. Further washing with DPBS/0.1%Tween 20 preceded the addition of the secondary antibody Goat Anti Rabbit IgG H&L Alexa Fluor®488 (ab150077, Abcam plc). Samples were incubated at room temperature protected from light for 1 hour. The washing process was repeated and the staining of the actin filament and the nucleus proceeded as previously explained.

A confocal microscope (Nikon A1+; Nikon Instruments), equipped with an immersion lens with 40x magnification, was chosen to image all the samples. Images were acquired via NIS

software, utilising predefined channels, DAPI, Alexa 488 and Alexa568. for DAPI, vinculin and collagen type 1, and phalloidin respectively.

2.5.5 Cell fixation for SEM

The samples for SEM analysis were collected at day 1, 7 and 21. As preparation for the fixation step, cell media was aspirated and the scaffolds washed with DPBS. Fixation was done by immersing the samples in a solution of 2% glutaraldehyde (TAAB Laboratory and Microscopy) for 10 minutes. Washing of samples included two immersions in Sorensen's Phosphate Buffer (TAAB Laboratory and Microscopy), each one with a duration of 15 minutes. Finally, samples were dipped in DPBS and then dehydrated. Dehydration was achieved by immersing samples in, 25%, 50%, and 75% ethanol (30minutes each) finalizing with 100% ethanol (2 x 1 hour).

2.2.4 Scanning Electron Microscopy (SEM)

For the SEM observation, unseeded samples were mounted on aluminium stubs and fixed using carbon tape and gold coated. The coating was performed with 5-15nm gold particles using a sputter coater (Polaron SEM Coating Unit). Following fixation and dehydration, the seeded samples were subjected to critical point drying with carbon dioxide in a Baltec Critical Point Dryer (Leica Geosystems Ltd). These specimens mounted on an aluminium stub with Achesons Silver Dag (Agar Scientific) and then dried overnight. Imaging was done on a Tescan Vega LMU SEM (Tescan), housed within EM Research Services, Newcastle University. The images were collected with the equipment's software, ATLAS (Tescan).

2.5.6 Osteogenic differentiation assessment

Osteogenic differentiation was determined by measuring the production of alkaline phosphatase (ALP). Culture media was removed, the scaffolds washed with DPBS and then fixation performed as in 2.4.4. Two additional washes with DPBS preceded the sample

alkalinisation with water/0.1M Tris solution (Sigma Aldrich), which was then replaced by 1ml of liquid yellow ALP solution (Sigma Life Science). Samples were incubated for 30 minutes at room temperature and light-protected. From each well, 100 μ L of solution were pipetted into a 96-well plate (in duplicate). The ALP activity was quantified by measuring the absorbance of the solution, using the same spectrometer as in 2.5.2. The reading was performed at 405nm, and the results plotted based on the values obtained from a standard curve. The calibration curve was created by placing different concentrations of 0.1 M Tris/ALP into a 96-well plate.

2.5.7 Assessment of Calcium deposition

To investigate cell mineral deposition, a staining for Calcium was applied. Samples from the ALP assay were washed in DPBS, stained with 1mL of Alizarin Red solution (Sigma Aldrich), and incubated at room temperature for 15 minutes. The staining was removed and samples were intensively washed with deionised water. Prior to imaging, scaffolds were dried overnight. Imaging of the samples was executed with a stereomicroscope equipped with a digital colour camera (Leica Microsystems). The images were obtained via the supplier software (LAS; Leica Microsystems) and used for visual observation, and site marking for the quantification step. Quantification was performed with XPS (Thermo Scientific K-Alpha XPS spectrometer), using a standard protocol. Briefly, samples were fixed to a clean stainless-steel plate and immobilised using copper clips. Scans were acquired with 0.4 eV steps, pass energy 150 eV, a dwell time of 10ms and averaged over 20 scans. Three positions in the sample were analysed, each consisting of a circle with a diameter of 400 μ m. Data were analysed with CasaXPS software.

2.5.8 Statistical analysis

The statistical analysis of data was performed on Graph Pad Prism 7, expressed as mean with a deviation based on group variability. With exception of the mechanical properties as printed

where $n=5$, the mean values and standard deviations were determined from 3 samples per time point, including the positive control. 2-way ANOVA with Tukey post-hoc multiple comparison, was used for levels of statistical significance of $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (****).

3 Results

2.1 Degradation behaviour during processing and after PBS immersion

2.1.1 Structural changes

SEM studies, shown in Figure 1, display clearly the presence of AW particles and AW fibres in each of the composites (PLLA/AWp and PLLA/AWf) while PLLA parts were smooth and absent of inclusions. On figure 1 lack of bond between filler and matrix on biocomposite filaments is evident. After 8 weeks of immersion in PBS no changes in the structure were detected.

Figure 1 - SEM image of filament and printed samples before and after 8 weeks of degradation in PBS. Circled area displaying a particle and a fibre of AW inside the respective composite filaments.

XRD of PLLA granules shows the presence of peaks typical from semi-crystalline PLLA alongside both structural phases, α and α' [20]. The spectra of the printed part is typical of an

amorphous structure, with only a residual peak of α phase. For the composite scaffolds, the result was identical (Figure 2).

Figure 2 - XRD pattern of PLLA granules (a) and printed PLLA scaffold (b).

^1H NMR DOSY measurements indicated the polymer size across all processed polymer materials was approximately equal (Fig 3). Measurements of the material in dilute solutions showed processing has led to a decrease on polymer size compared the raw unprocessed material (R_H CDCl_3 2.2 nm). Comparing all subsequent samples indicated processed PLLA R_H CDCl_3 1.45 ± 0.10 nm, despite whether it was aged or not, indicating the polymer material is stable and undergoes little degradation or mass loss over the 8 week studied period.

Figure 3 – Diffusion analysis of polymer samples in dilute CDCl_3 solution. a) The measured diffusion ($\text{m}^2 \text{S}^{-1}$) of PLLA and solvent proton peaks. b) Calculated average polymer hydrodynamic radii (in nm).

Values for the mechanical properties as printed are present in table 1. The flexural strength of the composites as printed differed, with PLLA/AWf attaining a significantly higher average yield and modulus compared to PLLA and PLLA/AWp ($p<0.001$ and $p<0.01$ respectively).

Table 1 – Mechanical properties of scaffolds as printed expressed in terms of mean $\pm$ SD for $n=5$.

Mechanical properties	3-point bend test	
	Yield point (MPa)	Effective modulus (GPa)
PLLA	16.6 $\pm$ 10.4	0.9 $\pm$ 0.3
PLLA/AWp	22.8 $\pm$ 4.5	1.0 $\pm$ 0.1
PLLA/AWf	29.4 $\pm$ 5.3	1.5 $\pm$ 0.2

2.1.2 Physicochemical changes and Ion leaching

No changes were found for the flexural strength and modulus after 8 weeks in PBS (figure 5a and 5b respectively). No mass loss was observed throughout the study (sup figure 2), which is in accordance with the results on figure 3. The pH values between samples was different at determined time points, however no trend was found for the changes (figure 5c). Despite the random variation along the study (7.2 to 7.6), the values are similar to the one of physiological conditions (~ 7.4)[21]. The ion leaching analysis is represented on figure 5d and 5e, with major observations noted at week 1 and week 2.

The ion leaching analysis is represented on figure 5d and 5e, with major observations noted at week 1 and week 2. An increase in Ca (figure 5d) and Mg (figure 5e) was readily detected at week 1 for PLLA/AWf, with Ca and Mg concentrations of 86 and 23 μ M, respectively. This value was significantly higher compared to the PLLA sample (11 μ M Ca and 4.5 μ M Mg) but was more similar to that of the PLLA/AWp sample (78 μ M Ca and 12 μ M Mg). At week 2 the same trend was observed, with PLLA/AWf showing the highest values (51 μ M Ca and 12 μ M Mg), PLLA/AWp showing lower concentrations (23 μ M Ca and 4.3 μ M Mg), and PLLA the lowest concentrations (5.7 μ M Ca and 1.7 μ M Mg). Past week 2, all values

remained basal and stable and no significant differences between samples were found.

Figure 5 - Flexural properties of scaffolds and ion leaching into PBS (week 1, 2, 4, 6 and 8 after degradation in PBS). a) Yield point; b) Effective modulus; c) pH variation of PBS solution; d) e) content of Ca and Mg, respectively. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).

2.2 Bioactivity assessment - Apatite deposition: morphology and calcium phosphate ratio (CaP)

The presence of bioactivity (figure 6) was only observed for composite samples. A clean surface for the PLLA samples was observed at all time points. Both composite samples presented mineralised deposits at the surface from day 1 to day 21. The detection of Ca and P using EDS (table 2) allowed the estimation of the CaP of the precipitates. The pH of the

solution (figure sup X) was similar for all samples with exception of day 7 when the PLLA/AWp had a higher pH than the PLLA. This was not considered significant.

Table 2 - CaP ratio of deposits detected at the surface of biocomposite samples. No deposits found for PLLA, with values registered as 0.

CaP (mean $\pm$ SD)	PLLA	PLLA/AWp	PLLA/AWf
1	0 $\pm$ 0	0 $\pm$ 0	1.5 $\pm$ 0.5
7	0 $\pm$ 0	1.57 $\pm$ 0.1	2 $\pm$ 0.1
14	0 $\pm$ 0	1 $\pm$ 0.7	0.78 $\pm$ 1.1
21	0 $\pm$ 0	1.56 $\pm$ 0.5	1.52 $\pm$ 0.5

Figure 6 - SEM analysis of samples and SBF solution after immersion for 21 days. Micrographs of composite samples show deposits of material at the surface.

3.3 Biological assay – Cell viability, osteogenesis and mineralisation

An increase in the cell number was registered for all samples up to day 14 followed by a stabilisation (figure 7a). PLLA/AWf and TCP samples showed significantly higher values for day 7, 14 and 21 ($p < 0.0001$) compared to PLLA/AWp and PLLA (ns). Cell viability was similar for all scaffolds at day 1 and day 3, as observed on figure 7b. Live cells seem attached from day 1 and back to normal morphology by day 3.

Figure 7 – Proliferation of cells seeded on PLLA and PLLA/AW scaffolds compared to tissue culture plastic via MTT assay, and cell viability using Live/dead. a) Estimation of cell number using MTT absorbance values and a pre-made standard curve. ***denotes significance at $p < 0.001$, and **** $p < 0.0001$. b) Cell attachment and viability at day 1 and day 3. Green indicating living cells and red dead cell. c)

Cell adherence after 24h, and full tissue formation after 21 days were assessed (Figure 6), with PLLA/AWf demonstrating a multilayer formation and an apparent higher cell density, supported by figure 7a and 7c. In terms of morphology, at day 1 cells are grouped and have a fibroblastic shape[22]. The morphology evolved to spindle-shape at day 7, a finalised as cobblestone-like, at day 21[23]. Dense tissue formation prevented a better observation.

Figure 8 - Evolution in cell morphology and attachment on the studied samples. SEM images in grey scale, on the right for each type of sample.

3.3.2 Osteogenic differentiation and cell mineralisation

The ALP activity (figure 9a) did not differ between samples apart from day 1 where PLLA and PLLA/AWp had significantly higher production than PLLA/AWf and TCP. From day 7 onwards samples did not differ between each other at any time point. When analysed individually, the ALP values for each sample did not change with time, with exception of PLLA which experiences a significant drop between day 7 and day 14. Figure 9b shows the presence of mineralisation on top of the cells in both composite scaffolds, and for PLLA/AWp octacalcium phosphate apatite (flake-like form)[24,25]. XPS analysis of the samples surface confirms the presence of calcium for composite samples only, with PLLA presenting a residual value due to equipment artefact. Calcium content was not significantly different between composites at any time point. XPS and EDS analysis were not performed on TCP samples due to the equipment limitations.

The visual presence of mineralisation was detected with alizarin red staining (figure 9d) at day 21. All samples presented red staining, but not the TCP. Presence of Ca and P was confirmed by EDS analysis (table 3), however only for the composite samples. The measurement were taken at sites where deposits were found on top of the cells to separate them from what could be carbonated apatite layer from the samples. Due to the lack of observable mineralisation on cells at day 1, it is assumed that this values are associated with the sample rather than the cells. PLLA samples reported an atomic % of 0 (zero), while the composites presented values within the range of calcium deficient apatite, with some samples being close to bone apatite (CaP of ~ 1.6). For the TCP, stereomicroscope images reported the absence of staining, consequently these were not included in the analysis (sup. Fig X).

Figure 9 – Osteogenic differentiation of seeded cells for polymer and composite samples. A) Alkaline Phosphatase activity measurement as osteogenesis indicator. b) SEM images of samples at day 21. Presence of octacalcium phosphate indicated by OCP on PLLA/AWp alongside cell with mineralised bone nodules indicating mineralisation. Ca layer on PLLA/AWf. c) XPS analysis of calcium content. d) Alizarin red staining of cell-seeded PLLA, PLLA/AWp and PLLA/AWf scaffolds. Micrographs of samples collected at day 21. Red stain indicated presence of calcium at the sample surface (bar 5mm). Statistical analysis between samples at each time point, $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).

Table 3 – CaP ratio of samples per time point. Values presented as mean $\pm$ SD for $n=3$.

CaP (mean $\pm$ SD)	PLLA	PLLA/AWp	PLLA/AWf
1	0 $\pm$ 0	1.71 $\pm$ 1.6	0.74 $\pm$ 0.8

7	0 ± 0	0.84 ± 1	0.92 ± 0.2
14	0 ± 0	1.21 ± 0.6	0.39 ± 0.3
21	0 ± 0	1.07 ± 0.6	0.61 ± 0.7

The confocal analysis (figure 10) supports these data; however at day 21 the thick tissue does not allow for further observations of the morphology. The staining of collagen type 1 also acted as a marker for cell differentiation into osteoblasts. The increasing concentrations of collagen type 1 with time (stained green) suggest that cells are differentiating into osteoblasts in all tested samples[26].

Figure 10 - Confocal images, using blue staining for nucleus (DAPI), red for cell body (Phalloidin) and green the actin filaments representing adherence (Vinculin) and collagen type 1.

4. Discussion

4.1 Processing of composite: from filament production to printing

In this study we demonstrate the successful optimisation of both filament making and printing for both particle and short fibre reinforced composites. The homogenous distribution of the filler was attained from the initial blending of the material and its mixing inside the

extruder[5]. Fibre alignment on the PLLA/AWf was expected since fibre alignment on the printing direction due to shear forces inside the nozzle, is well reported for both long and short carbon and glass fibres[27–29]. The printing process made the polymer structure amorphous (Figure 2), which could be associated to the fast cool down of material[20,30]. This structural change in crystallinity could lead to higher degradation rate since the crystal segments are chemically more stable than the amorphous ones. In applications where mechanical support is not essential but biodegradability is sought, an amorphous structure might be desirable.

4.2 Mechanical properties and degradation profile

Both the average modulus and average yield stress were broadly consistent over the 8 week period for all three materials. The addition of the short AW fibres slightly increased the flexural strength of the scaffolds as printed angular shaped particles are preferred particle shapes for reinforcement as they act as interlocks, decreasing the disruption between matrix and filler during mechanical testing. Unless used in high ratios, >30% [31,32], the use of bioceramic particles as reinforcement will not provide an improvement of mechanical properties, being used to stimulate a response[31,33]. The slight improvement in initial mechanical properties of the fibre reinforced composite could be associated with factors such as fibre wetting. Fibres can retain heat which promotes material diffusion, resulting in improved bonding with the matrix, therefore resulting in stronger material[27,34].

All samples remained stable under PBS immersion, with no mass loss. This suggests the sample is not degrading in bulk. The pH oscillated but stayed within the physiological range, ideal for implantation[21]. The NMR data (Fig. 3) suggest that the material is not suffering chemical degradation, which is supported by previous studies[35,36]. These data are supported by the absence of changes in the mechanical behaviour of the parts and lack of defect sites and cracks that are common for PLLA hydrolytic degradation process[21].

The ion release profile changed significantly only for the composites, suggesting that Ca and Mg are easy to release from the structure, and therefore their presence is noted from week 1[37]. The Ca and Mg are only present in the AW, suggesting they are being leached from exposed fibres and particles. Once they are consumed, the values drop, in this case by week 4, and are maintained relatively stable until the end. This suggests that the fibres are still exposed and that the Ca and Mg are consumed[37]. This release is gradual suggesting that there are multiple fibres, possibly of different sizes or with different amounts exposed[35,38]. The lower leaching for the AWp samples suggests that less of the AW is present at the surface of these composites. Smaller particles with a less rounded shape may be easier to submerge into the matrix as a result of the high shear stresses at the walls when the filament is extruded.

4.4 Bioactivity study

The bioactivity of the composite is dependent on the exposure of ceramic at the surface. A minimum bioceramic loading of 20% is advised in order to attain bioactivity[5]. From the SEM and EDS analysis, the deposits on top of both composites were calcium deficient apatite, with CaP ratio varying between 0.4 and 1.7, the latter close to bone apatite (1.67)[39]. This is likely to enhance the osteoconductive behaviour and improve implant-bone bonding for both materials[40,41]. Apatite was also observed in both cauliflower and flake form. Cauliflower apatite is commonly observed as a product of bioactivity, but flake apatite is harder to produce once it demands the growth in specific crystalline planes[42,43].

4.5 Influence of samples in cell behaviour

4.5.1 Cells growth and morphology

The PLLA and PLLA/AWp materials showed no significant differences in terms of cell viability and proliferation, whilst PLLA/AWf and TCP values were significantly higher from day 7 onwards (Fig 7a). Cells grew and proliferated in all types of samples, as observed in figure 8. Moreover, cells presented a fibroblastic morphology at day 1 (typical of hMSCs), which evolved into pre osteoblastic at day 7. By day 21, cells evidenced a polygonal cell shape typical of osteoblasts [44–47] and ECM production (Fig 10). High proliferation rate is common on TCP since its surface is treated to support cell growth [48]. As summarised above, main differences between samples consists in higher proliferation rates for PLLA/AWf rather than variation on cell morphology or production of collagen type I. Since both composites contains the same filler composition (AW precursor), degradability and higher ions release in PLLA/AWf (Fig 5) may stimulate cell growth while AW precursor itself promotes differentiation and ECM deposition.

4.5.2 Cell differentiation and biomineralisation

In terms of ALP concentration the samples only differ between each other at day 1, where PLLA and PLLA/AWp present a higher level of ALP production per cell (Fig 9a) compared to PLLA/AWf and TCP. PLLA and PLLA/AWp appear to be more osteoinductive at first contact. However, all three cultures produced differentiated cells by day 21 (Fig 8 and Fig 10, respectively). Osteogenesis can also be detected by increase in the production of collagen type I. The confocal imaging (Fig 10) shows the presence of collagen type I increases significantly from day 7, for all types of samples. The production of collagen type I and type II by stem cells prior to differentiation is known[19,47,50]. However, depending on the cell line, collagen type I gene is upregulated and collagen type II is downregulated during osteogenesis, after 7 and/or 14 days [26,45,47,50].

Following the differentiation of cells into osteoblasts, mineralisation occurs, characterised by a drop in ALP values and production of calcium deposits that are visible around the cells

trapped in ECM [22,50]. Although ALP is a common marker for MSCs differentiation, it does not predict the mineralisation stage or rate by itself [51–53]. Cells seeded on all samples produced ALP, but mineralisation was missing from the TCP control, and the mineral shape differed between the polymer and composite samples. PLLA/AWf showed consistent plate-like structures of CaP (Figure 9b), whilst PLLA and PLLA/AWp evidenced small calcium deposits on top of the cells. ALP provides free phosphate to cells, for the production of hydroxyapatite and in parallel inhibits the bone matrix formation [51]. The ion leaching profile indicated that PLLA/AWf is the only sample showing longest period of ion leaching (up to week 4). PLLA/AWp presented significant ion leaching only in the first week of the study, and for PLLA it was always residual. (Fig 5). High levels of calcium at the surface were also detected by alizarin red staining, where a denser coating is visible for PLLA/AWf, compared to PLLA and PLLA/AWp. If Ca is available then phosphate will also be available. It may be that the presence of phosphate suppressed the production of ALP on the composites, but with a stronger effect on PLLA/AWf, due to the longer exposure to it. Its absence in PLLA, and the limited amount in PLLA/AWp, allowed for initially higher ALP levels. In essence, we argue that ALP was not produced in the PLLA/AWf cultures because phosphate was already available in the media, but for PLLA/AWp and PLLA cultures it wasn't as prominent. Previous studies show that the ingestion of HA particles smaller than 50 μm could accelerate the osteogenic differentiation of human MSCs derived from adipose tissue [54]. This is due to the great availability of Ca and P ions that, when in contact and digested by the cells, trigger cell signalling pathways that would lead to osteogenesis and mineralisation. From the bioactivity assay and the alizarin red staining, the existence of two sources of calcium in the composites has been demonstrated: from the material and the cells, and we consider that both play an important part in cell differentiation and mineralisation [22,26,47,55,56].

4.3 Suitability as device for critical size defects

Healing of bone fractures and reconstruction of critical-size defects is a significant challenge, since these do not heal spontaneously over the patient lifetime. Bone is a self-healing organ, however, this type of defect is highly dependent on 2 factors: 1) the surrounding tissue health, namely its vascularisation to allow nutrient access; 2) the MSCs present in bone marrow and periosteum, which are the osteoprogenitor cells. Secondary mechanisms influencing these cells include osteogenesis, osteoinduction and osteoconduction. At the fracture site, osteoinductive factors are delivered by the vascular network. In defects where vascularisation is limited, an external stimulus must be applied to induce the cells differentiation[57]. The fibre reinforced composites could be especially attractive to solve this problem due to their ion leaching profile. The leached ions from the PLLA/AWf samples, Ca^{2+} and Mg^{2+} , are known for their effects/contributions towards bone growth and maturation [61,62]. Mg^{2+} is not only essential for bone metabolism and mineralisation of calcified tissues, but also stimulates osteogenesis. Ca also contributes for cell differentiation and proliferation, and later favours ECM mineralisation[63]. In terms of osteoconduction within the human body, the osteoconductive platform is created via the formation of hematoma and cartilage callus, which then supports the osteoblast growth at the defect site[57]. In this work we present a new process for the development and manufacturing of osteoinductive, and osteoconductive, resorbable biocomposites, without the use of additives, surface treatment or differentiation media.

Clinically, the most commonly used treatments for critical size defects are bone grafts, distraction osteogenesis (DO) and induced membrane technique (Masquelet), but all have their own limitations. Autologous bone grafts are the gold standard solution however, difficulties arising from the harvesting procedure and associated with the amount and quality

of bone hinder their usage in larger defects[58]. DO relies on the use of metal plates and screws to keep the bone cut in place throughout the healing process[57]. This does not eliminate the problem of stress shielding and possibly implies a second intervention to remove the plates. It also cannot be applied to individuals suffering of low bone density or osteoporosis[59]. The Masquelet technique has proven advantageous in defects up to 25 cm but it implies two interventions and the use of both bone cement and bone grafts. This can hinder the patient recovery, which includes 8 weeks for the first step alone[60]. The composites developed proved to be osteoinductive and osteoconductive, inducing the hMSCs differentiation and mineralisation, alongside cell growth and proliferation, therefore comprising the main qualities found in human bone grafts. They also are bioresorbable, therefore do not need a second intervention for their removal, as in DO and the Masquelet procedure. In terms of mechanical support when implanted, the scaffolds proved to be intact after 8 weeks in degradation media, which would be sufficient for the bone healing process, and would match the period covered on the Masquelet first step, which aims the stabilisation and initial bone repair[57,60]. Finally, from a commercial and manufacturing point of view, FFF allows the production of samples with complex shapes but without the use of chemicals and other additives, which facilitates the process scale up to industrial level. This would allow the production of more devices faster and at a lower cost.

5. Conclusions

Three types of samples were studied, PLLA and PLLA reinforced with AW particles and PLLA reinforced with AW fibres to assess their suitability as scaffolds for bone repair in critical size defects. Both polymer and biocomposites showed a good biocompatibility, and mechanical properties equivalent to cancellous bone, under degrading conditions, with the fibre reinforced composite performing better as produced. No mass loss is detected indicating

the sample is not degrading in bulk up to 8 weeks therefore it could be used for long term applications. Its degradability can be tuned by increasing the AW content[35,38].

All samples promoted osteogenesis, however the biocomposites induced a higher degree of biomineralisation. The presence of calcium on top of the biocomposite samples indicated the material ability to induce apatite deposition, which was detected with SEM and alizarin red staining. This is a valuable asset since once implanted there would be two sources of calcium, which would directly accelerate bone repair and regeneration. Both composites have potential for use in critical size bone defects, with the AWf composite showing greater levels of ion release, stimulating more rapid cell proliferation and greater levels of mineralisation during cells assays.

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