Short phosphate glass fibre reinforcement of PLLA to promote bone mineralisation

Priscila Melo¹, Emma Tarrant², Thomas Swift³, Anthony Townshend⁴, Matthew German⁴, Ana-Marina Ferreira¹, Piergiorgio Gentile¹, Kenneth Dalgarno¹

¹ School of Engineering, Newcastle University, Claremont Road, NE1 7RU, Newcastle Upon Tyne, United Kingdom.

² Institute for Cell and Molecular Biosciences, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne, NE2 4HH, United Kingdom.

³ School of Chemistry and Biosciences, Faculty of Life Sciences, University of Bradford, Bradford, BD7 1DP, United Kingdom.

⁴ Centre for Oral Health Research, School of Dental Sciences, Newcastle University, Newcastle Upon Tyne, NE2 4BW, United Kingdom.

Abstract: The clinical application of composites seeks to exploit the mechanical and chemical properties of materials which make up the composite, and in researching polymer composites for biomedical applications the aim is usually to enhance the bioactivity of the polymer, whilst maintaining the mechanical properties. To that end, in this study medical grade Poly(L-lactic) acid (PLLA) has been reinforced with short phosphate-based glass fibres. The materials were initially mixed by melting PLLA granules with the short fibres, before being extruded to form a homogenous filament, which was pelletized and used as feedstock for compression moulding. As made the composite materials had a bending strength of 51MPa $\pm$ 5, and over the course of eight weeks in PBS the average strength of the composite material was in the range 20-50 MPa. Human mesenchymal stromal cells were cultured on the surfaces of scaffolds, and the metabolic activity, alkaline phosphatase production and mineralisation monitored over a three week period. The short fibre reinforcement made no significant difference to cell proliferation or differentiation, but had a clear and immediate osteoinductive effect, promoting mineralisation by cells at the material surface. It is concluded that the PLLA/PGF composite material offers a material with both the mechanical and biological properties for potential application to bone implants and fixation, particularly where an osteoinductive effect would be valuable.

Keywords: short fibre composite, bone, compression moulding, osteoinduction.

1. Introduction

Demand for new orthopaedic technologies has risen due to the growing number of bone defects caused by pathologies such as osteoporosis. In the United States an estimated 10 million individuals above 50 years old are diagnosed with osteoporosis every year, and 1.5 million individuals suffer from osteoporosis related fractures[1], with women at a higher risk than men. Overall, the healthcare cost of osteoporotic fractures is estimated at \$13.8 billion per year, not including the extra resources the patient will need further on, which are twice as much as for a normal patient[1]. Advances in medicine have provided some solutions for osteoporotic fracture fixation, but problems such as re-fracturing, either derived from stress shielding in metal plates, or simply due to bone weakening from the first fracture, remain unresolved[1,2]. It is known that patients that had a fracture, even if low trauma, will be prone to suffer from another fracture. In the case of hip fracture, 10-14% of the individuals will suffer a new one each year. Therapies such as calcium and vitamin D administration, alongside other preventive drugs, are used as general practice, in some cases, site guards such as the hip external protector[1].

For the treatment of osteoporotic fractures, a device with both good mechanical properties and the ability to stimulate a local osteoinductive response would be ideal. Bone grafts, considered the gold standard, are restricted to small defects, and are incapable of being placed in load bearing sites[1,3,4]. Resorbable devices made of Poly(lactic acid) (PLA) or Poly lactic-co-glycolic acid (PLGA) have been assessed[5], but do not generate an osteoinductive response. Resorbable biopolymer-bioceramic composite materials have the potential to provide the mechanical properties required, together with an osteoinductive response from the bioceramic phase [6]. Polymer-ceramic composites can be particulate, short fibre, long fibre or weave, dependent upon the form of the ceramic phase. However, reviews of composites for biomedical applications make it clear that particulate reinforcement dominates

[6,7], with a limited number of studies using long fibre phosphate glass reinforcement [8–10]. For bone applications, phosphate and hydroxyapatite are the most common materials used, as fibres and particulate, respectively as these materials are known to be osteoinductive [8,9] but, for polymer composites which are load bearing, there seem to be no studies which demonstrate any kind of osteoinductive effect prior to the polymer degradation. As the degradation of load bearing polymers takes weeks to months, this means that the osteoinductive effect occurs long after implantation, well beyond the point at which it would be of most value.

In this study we explore a novel approach to create a load bearing osteoinductive material, based on reinforcement of PLLA with a Ca-doped, phosphate glass short fibre. The aim was to explore whether or not the use of short fibre allowed more filler to be present at the surface of the composite, therefore resulting in a rapid resorption of the surface fibres. The resorption would lead to Ca availability at the surface, then then generating an immediate osteoinductive response.

2. Materials and Methods

Calcium doped PGFs were supplied by Glass Technology Services (Sheffield, United Kingdom), the mol% of the PGF used is 55P₂O₅- 5Na₂O-16CaO-24MgO, with density of 2.55g/cm³, average diameter of 20 µm (with a standard deviation of 10 µm) and an average fibre length of 166 µm (with a standard deviation of 64 µm). Medical grade PLLA (Purasorb PL 38, Corbion - Purac Biomaterials) with average density of 1.24g/cm³ was used as matrix.

2.1 Production of composite samples

2.1.1 Material blending

A set of composite rods, measuring 200 x 3 x 4mm, were produced via melting using an aluminium mould with rods of 1.90±0.02g of PLLA granules and 0.10±0.02g of milled PGF.

Half the mass of the PLLA was poured in each groove and melted at 220°C for a homogeneous layer. PGF and remaining PLLA were then added and blended with a spatula. The material was melted at 220°C for 15 minutes, then left to cool down at room temperature then pelletized (Rondol Technology Ltd, United Kingdom) to create granules.

2.1.2 Homogenization of fibre distribution

The granules were extruded using a twin screw system (Rondol Technology Ltd, United Kingdom) to distribute the fibres within the polymer matrix. For the extrusion process, the peak temperature was 190°C, with the temperature at the die 160°C. The material was inserted in a haul-off machine and pulled at 0.7mm/s, for a feed speed 20 ± 5 rpm and extrusion speed 60 ± 5 rpm. The resultant filament was 2 ± 0.1 mm in diameter, and was pelletized.

2.1.3 Sample preparation: compression moulding

Samples ($n=3$) were prepared via compression moulding: discs of 8 mm diameter and 2 mm thickness for the biological characterisation; discs of 8 mm diameter and 4 mm thick for physicochemical characterisation; and bars, 40 x 3 x 2 mm to test the flexural strength. The pellets obtained from section 2.1.2 were poured into aluminium moulds (0.4 g and 0.2 g for bars and discs, respectively), pressure applied through an upper mould to close the system. An iron bar weighing 935 g was placed on top of the counter mould to create pressure and the whole assembly placed in the oven at 190°C (PF 60, Carbolite Gero Ltd) for 2 hours. Different moulds were used to produce each shape at different sealing pressures, 6.4 kPa and 94 kPa for the discs and bars, respectively. Finally, the iron weights were manually pressed to release air bubbles and the mould left to cool down inside the oven overnight.

2.2 Material characterisation

2.2.1 Flexural mechanical properties

The mechanical properties (n=3 per material) of the specimens were assessed through a 3-point bend test (1 mm/min cross-head speed, Instron 5567, Bucks, UK) with a span length between the lower supports of 20 mm on the test fixture (2810-400, Instron).

2.2.2 X-Ray Diffraction (XRD)

Crystallinity changes were studied using a PANalytical X'Pert Pro MPD, powered by a Philips PW3040/60 X-ray generator and fitted with an X'Celerator detector. Diffraction data was acquired by exposing powder samples to Cu-K α X-ray radiation, with a characteristic wavelength (λ) of 1.5418 Å. X-rays were 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)

Polymer hydrodynamic radii were determined via ^1H proton measurements using a Bruker Avance III 400 MHz nuclear magnetic resonance (NMR) spectrometer, using 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. Samples were analysed using a bipolar gradient pulse, and 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[10]. 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 ($5.28 \times 10^{-4} \text{ kg m}^{-1} \text{ s}^{-1}$)[11]. 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 assays

2.3.1 Mass loss, pH change and ion release

Disc shaped specimens (n=5 per material) were weighed using an analytical balance (AE240, Mettler Toledo, UK) and then placed in individual glass vials containing fresh Dulbecco's phosphate buffered saline (PBS, pH 7.4, Gibco, Thermo Fisher Scientific, UK) and stored at 37°C (Gallenkamp, UK) for 8 weeks. The specimens were placed into fresh PBS weekly. At weeks 1, 2, 4, 6 and 8 the specimens were removed from the PBS, the PBS reserved for further analysis and prior to placing in fresh PBS, the specimens were blotted dry with absorbent tissue and re-weighed. The pH of the reserved PBS was then measured using a pH electrode (59001-82A, Cole-Parmer, UK) connected to a pH meter (Orion 4 Star, Thermo Fisher). Next, the PBS samples were analysed by inductively coupled plasma mass spectrometry (ICP-MS) to determine elemental composition on a Thermo X-series instrument operating in collision cell mode (using 3.0 mL/min 8% H₂ in He as collision gas). The samples were diluted 5-fold in 2.5% (w/v) high-purity HNO₃ (Merck) containing 20 µg/L silver (Ag) and platinum (Pt) as internal elemental standards. For each sample, sodium (²³Na), magnesium (²⁴Mg), phosphorus (³¹P), potassium (³⁹K), calcium (⁴⁰Ca), manganese (⁵⁵Mn), iron (⁵⁶Fe), cobalt (⁵⁹Co), nickel (⁶⁰Ni), copper (⁶⁵Cu), zinc (⁶⁶Zn), silver (¹⁰⁷Ag), and platinum (¹⁹⁵Pt) isotopes were monitored sequentially using the peak-jump method (100 individual reads of 20 ms on each isotope, across 3 channels of 0.02 atomic mass unit separation, each in triplicate). Elemental concentrations were determined by comparison with matrix-matched standard solutions of known elemental composition (0 – 5,000 µg/L) analysed within the same run.

2.4 Cell studies on composites scaffolds

2.4.1 Cell seeding method

TERT Human Mesenchymal Stromal Cells (hTERT-MSCs) Y201, an immortalized cell line [12], were used for cell tests (passage 87). Scaffolds were sterilised with 70% ethyl alcohol solution (Sigma Aldrich, UK) for 15 minutes, followed by UV irradiation on a 302 nm waveband (EL Series UV Lamps, Ultra-Violet Products Ltd.) for 40 minutes. The discs were then washed with Dulbecco's PBS (DPBS; Sigma Aldrich, UK) and placed into 12 well plates. hTERT MSCs were seeded in triplicate on top of the discs at a density of 10 000 cells/disc, before incubation at 37°C in a humidified atmosphere of 5% CO₂. A set of discs (n=3) without cells was used as control for the calcium mineralisation. 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 used as a positive control. Cells were cultured up to 21 days.

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

The metabolic activity of cells was measured in triplicated using an MTT kit (Thiazolyl Blue Tetrazolium Bromide; Sigma Aldrich, UK) after 1, 3, 7, 14 and 21 days. The MTT solution was prepared according to supplier instructions (5mg/mL in DPBS) and mixed with serum-free DMEM deprived of phenol red (Gibco; Thermo Fisher, UK), reaching a final concentration of 0.5 mg/mL. After 4 hours incubation at room temperature with protection from light, the media was removed and replaced with 400µL of isopropanol. Plates were agitated for 30 minutes to dissolve the formazan crystals. Afterward, 200µL of the dissolved formazan was transferred to 96-well plate and absorbance was measured with a spectrometer (ELx800; BioTek Instruments, UK) at 570nm. To estimate the cell number, a standard curve

was created by seeding hTERT-MSCs in different densities (0, 10 000, 30 000, and from there onwards up to 450 000 with a 50 000 increment).

2.4.3 Live and dead staining

Cytotoxicity was assessed at day 1 and day 3, through the use of a live/dead kit (Thermo Fisher, UK). The stock solutions were prepared in accordance to the manufacturer's recommendations, meaning a final concentration of 4 μ M of ethidium homodimer-1 (EthD1) and 2 μ M of calcein. Briefly, the media was removed from the wells and the samples were washed twice with PBS. The prepared solution was pipetted into the wells, followed by incubation for 30 minutes at room temperature, protected from light. Imaging was through an inverted confocal microscope (LSM 800; Carl Zeiss Ltd) which required the placement of each sample in a supplied plastic dish (seeding surface facing the lens), and the addition of DPBS to avoid sample drying. For the imaging, predefined laser wavelengths were applied, AF488 (410-546nm) for green (live) and AF546 (576-700nm) for red (dead).

2.4.4 Cell fixation and staining for confocal microscopy

Samples were collected at day 1, 7 and 21 for morphological observation via confocal microscopy. The cellular media was aspirated and samples were washed with DPBS. A solution of 4% paraformaldehyde (Sigma Aldrich) was added to the wells and incubated for 15 minutes at room temperature. Cells were washed 3 times with a DPBS/0.1% Tween 20 solution, immersed in 3%w/v Goat's serum/DPBS/0.1% Tween 20 and incubated for 30 minutes at room temperature. A primary antibody solution was added, consisting of 3% w/v Goat's serum/DPBS/0.1% Tween 20 and Vinculin (ABfinity rabbit; Thermo Fisher), on a ratio of 1:200. Samples were then incubated for 1 hour at room temperature, light-protected. Further washing with DPBS/0.1% Tween 20 preceded the addition of the secondary antibody (Alexa Fluor 488, donkey anti-mouse antibody; Life Technologies) which was prepared and incubated under the conditions described for the primary antibody. The washing process was

repeated and 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. After a washing cycle, the 4',6-diamidino-2-phenylindole (DAPI; Sigma Aldrich) was added and incubated at room temperature, light-protected, for 1 hour. Finally, samples were washed with DPBS/0.1% Tween 20 once, followed by DPBS wash step. Samples were imaged using confocal microscope (Nikon A1+; Nikon Instruments) with an immersion lens with 40x magnification. Images were acquired using the NIS software and predefined channel modalities, DAPI, Alexa 488 and Alx568.

2.4.5 Cell fixation for SEM

SEM analysis was performed on samples collected at day 1, 7 and 21. The cell media was aspirated and the specimens were washed with DPBS. Fixation was performed with a solution of 2% Glutaraldehyde (TAAB Laboratory and Microscopy) for 10 minutes followed by two immersions in Sorensen's Phosphate Buffer (TAAB Laboratory and Microscopy), each one with a duration of 15 minutes. Samples were rinsed in DPBS and dehydrated.

2.4.6 Scanning Electron Microscopy (SEM)

Specimens were mounted on aluminium stubs and fixed using carbon adhesives. The SEM analysis of the samples from the biological assay consisted on the sample dehydration followed by critical point drying and gold coating. Dehydration was carried out using, 25%, 50%, and 75% ethanol (30minutes each) followed by 100% ethanol (2 x 1 hour). A final dehydration step was performed with carbon dioxide in a Baltec Critical Point Dryer (Leica Geosystems Ltd). Fixed specimens were mounted on an aluminium stub with Achesons Silver Dag (Agar Scientific) and then dried overnight. The coating was performed with 5-15nm gold particles using a sputter coater (Polaron SEM Coating Unit). Samples were examined using a Tescan Vega LMU SEM (Tescan), housed within EM Research Services, Newcastle University. Digital images collected with TESCAN supplied software

2.4.7 Osteogenic differentiation assessment

Osteogenic differentiation was evaluated using Alkaline Phosphatase (ALP). Cellular media was removed and samples washed with DPBS. Sample fixation was performed using the method described in 2.4.4. A double washing step with DPBS was performed followed by alkalisation with Water/0.1MTris solution (Sigma Aldrich). The alkaline solution in the wells was replaced by 1ml of liquid yellow ALP solution (Sigma Life Science), followed by incubation for 30 minutes at room temperature and light-protected. 100 μ L of solution was then taken from each well and placed in a 96-well plate to quantify the ALP activity. The reading was performed at 405nm, with a standard curve created for calibration by placing different concentrations of 0.1MTris/ALP into a 96-well plate.

2.4.8 Assessment of Calcium Deposition

Samples used in section 2.4.5 were washed in DPBS twice and stained with 1mL of Alizarin Red (AR) solution (Sigma Aldrich) for the detection of Calcium. Incubation was at room temperature for 15 minutes. Samples were washed with deionised water several times until no red colour was observed and dried overnight at 60°C in a 5% CO₂ atmosphere. Samples imaging was performed on stereomicroscope equipped with a digital colour camera (Leica Microsystems). The images were acquired using the supplier software (LAS; Leica Microsystems). Quantification of calcium deposition at the surface of samples was performed with X-Ray Photoelectron Spectroscopy (XPS) on 1 sample per each time point. All the spectra were acquired with a Thermo Scientific K-Alpha XPS spectrometer at 0.4 eV steps, pass energy of 150 eV and a dwell time of 10 ms per 20 scans. The samples were mounted on a clean stainless steel plate and immobilised using copper clips. The spectra were repeated at three analysis positions on each sample (diameter of 400 μ m) with non-overlapping analysis areas. Data analysis was performed with the CasaXPS software.

2.4.9 Statistical analysis

The collected data was analysed with the software Graph Pad Prism 7 and expressed as mean with a standard deviation taken as measure of group variability. Mean values and standard deviations were calculated from 3 samples per time point, including the positive control. The results analysed using 2-way ANOVA with Tukey post-hoc multiple comparison, for levels of statistical significance of $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).

3. Results

3.1 Degradation behaviour during processing and after PBS immersion

3.1.1 Structural changes

SEM studies (figure 1) show a clean structure for the PLLA and presence of PGF in the composite. An alignment of the PGF can be seen for both filament and moulded samples. On the filament, the alignment occurs in the direction of extrusion and on the sample, perpendicular to the applied load in moulding. After 8 weeks under immersion in PBS, deposits could be observed for the composite samples, but no evidence of degradation could be seen in any of the sample types.

XRD diffraction of the PLLA as supplied and after compression moulding shows the presence of peaks typical of semi-crystalline PLLA and well as both structural phases, α and α' [13] (figure 2).

Figure 1 - SEM image of a cross-section of extruded filament, compression moulded sample and sample after 8 weeks of degradation. Arrow indicates load direction when compression moulded.

Figure 2 - XRD pattern of PLLA raw (a) and PLLA after compression moulding (b).

The hydrodynamic radii of unprocessed PLLA granules were found to be approximately 5 times larger than the compression moulded samples (Figure 3 c). However post these thermal processes the polymer appears to have remained stable for the first six weeks of the study with no change in polymer size or mass. In week 8 additional proton peaks began to appear (3.94 ± 0.95 ppm).

Figure 3 ^1H diffusion NMR data of PLLA samples with corresponding CDCl_3 (solvent) shifts.

Proton signal loss with magnetic gradient of a) raw PLLA compared to thermally processed samples, b) aged samples in PBS weeks 1 – 6. Solvent proton decays for each samples are represented by empty boxes. c) Determined particle hydrodynamic radii of all samples.

Figure 4 shows that the sample mass did not change significantly for either material along the course of 8 weeks in PBS, with the highest value reported being 0.2g for the polymer sample. The flexural strength did not significantly decrease for either material over 8 weeks ($P > 0.05$) with the composite specimens generally exhibiting the greater strength, although this was statistically significant at any time point.

3.1.2 Chemical changes and Ion leaching

The PBS solution pH (figure 5a) was significantly different between samples at weeks 1, 2 and 8. The polymer pH is relatively stable throughout the study while the composite experiences an initial decrease in pH followed by an increase from week 1 to week 4, and a stabilisation up to week 8. ICP-MS analysis was performed for Magnesium (Mg) and Calcium (Ca), on both samples (Figure 5b and 5c). The content of these 2 ions in the PLLA

PBS solutions (3-20 μM) was significantly lower ($p < 0.0001$) than that in the composite PBS solutions (50-1000 μM). Exposure of fibres on the composite was observed (Figure 5d).

Figure 4 – Mass loss and mechanical properties before and after degradation in PBS.

3.2 Biological assay – Cell viability, osteogenesis and mineralisation

Figure 6a, shows an increase in cell metabolic activity up to day 14 followed by a slight decrease at day 21, with no significant difference between cells cultured on the polymer or the composite. Estimated cell number is represented in figure 6c, suggesting that cells proliferated up to day 14 with significant difference on cell behaviour between the two materials. Cell viability was also similar for both polymer and composite, as observed at day 1 and 3 on the live dead assay (figure 6b) where live cells seemed attached at day 1 and back to their normal morphology by day 3.

Cell morphology was observed by confocal and SEM analysis, and tracked over 21 days of incubation (figure 7). There was no difference between both samples in terms of cell adherence after 24h, and full tissue formation after 21 days. At day 7, a lower number of cells was observed at the top of the polymer. The cell morphology (pointed by arrows) evolved from fibroblastic shape-like at day 1, to spindle-shaped at day 7. At day 21, some cells resemble cobblestone-shaped osteoblast but the presence of a dense tissue prevents a better observation[14].

Figure 5 - pH changes, ion leaching before and after degradation study and sample defects. a) pH variation of PBS solution. b) content of Mg and c) Ca in PBS solution collected from samples and original PBS; d) SEM micrograph of composite sample showing exposed fibres.

Figure 6 – Proliferation of cells seeded on PLLA and PLLA/PGF discs compared to tissue culture plastic (TCP) via MTT assay, and cell viability using Live/dead. a) Cell metabolic activity during 21 days measured. ***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) Estimation of cell number using MTT absorbance values and a pre-made standard curve.

3.2.1 Osteogenic differentiation and cell mineralisation

There were no significant differences in ALP activity between samples within each time point, but between time points significant differences were observed for cells cultured on the composite material. The ALP activity decreased significantly from day 1 to day 14 and remained at that value up to day 21, while the polymer remained stable. The micrographs shown on figure 8 contain spindle-shaped and cobble shaped cells, previously stated from figure 7. Deposits of material were detected for the composite sample but not for the polymer.

Figure 7 - Evolution in cell morphology and attachment on the studied samples. Arrows pointing out different cell morphologies, fibroblastic, spindle-like and cobble-stone. SEM images in grey scale, on the right for each type of sample and confocal images on the left. On the confocal images, blue staining for nucleus (DAPI), red for cell body (Phalloidin) and green the actin filaments representing adherence (Vinculin).

Figure 8 – Cell differentiation and mineralisation. a) Alkaline Phosphatase activity measurement as osteogenesis indicator. b) SEM images of cell morphology at days 7 and 21. Cells adhered and spread on the surface of both sample, with morphology evolving from spindle-like (red arrow) to cobble-stone (black arrow) form day 7 to day 21.

Figure 9a shows that the polymer samples did not stain for calcium at any time point. A gradual increase in the area covered with red was observed for the composite samples, with more area covered on samples that were cell seeded. The calcium and phosphate presence was firstly observed using EDS (supplementary information), and later quantified with XPS (figure 9b) confirmed the presence of calcium at selected locations on the surface of composite samples, seeded and non-seeded. For the polymer samples calcium was not detected, with the residual values registered being an artefact of the analysis itself, which was also confirmed by EDS (supplementary information). The phosphate content was similar for all samples with exception of day 1 where the composite samples seeded with cells presented a significantly higher percentage, compared to non-seeded samples and to the polymer. In terms of the evolution of phosphate content over the time period, the major difference observed was that the seeded samples showed a decrease in phosphate percentage from day 1 to day 7, which then stabilized until the end of the study. For the other samples the percentage of phosphate remained stable throughout the 21 days. The calcium phosphate ratio was determined from EDS (see supplementary information). Polymer samples registered values of 0 while for the seeded and non-seeded composite presented values ranging from within the 0 to 6.

4. Discussion

4.1 Mechanical properties and degradation profile

The key observations in terms of mechanical properties (Fig. 4) are (a) the crystallinity of PLLA did not change with the processing[13], (b) the presence of both α and α' phases, responsible for PLLA's improved mechanical properties is evident in the spectra[13], (c) the addition of the short PGFs did not significantly change the strength of the material, and (d) that for both the PLLA and the PLLA/PGF the strength was broadly maintained over the 8

week period in PBS. The consistency in mechanical properties was associated with effectively no mass loss, suggesting that no bulk degradation is taking place. However, the NMR data (Fig. 3) suggests that at week 8 the polymer is beginning to show signs of chemical degradation with new functional groups starting to appear in the NMR profile. The week 8 hydrodynamic radii could be larger either because changing functional groups are causing it to swell, or because it is no longer a pure solution but contains aggregates: either case would suggest the onset of degradation (further details in the supplementary material). The ion release profile (Fig. 5) for Mg and Ca in the composite sample was similar over the 8 week time course: an increase in the first week, followed by a gradual reduction. The gradual leaching of the ions is considered to arise from fibre degradation. Even though the sample mass is not declining, the surface exposed fibres can be accessed. The pH of the polymer samples did not change significantly, but for the composite sample, an initial decrease in pH solution was observed, and we consider this to confirm the PGF degradation, which causes the release of phosphate ions, which later form phosphoric acid (H_3PO_4).

4.2 Biological properties

The PLLA and PLLA/PGF materials showed no significant differences in terms of cell viability and proliferation (Figs. 6 & 7). Cells grew and proliferated on both types of samples, and the morphology of cells changed from fibroblastic shape at day 1 (typical of hMSCs), to pre-osteoblastic at day 7, and finally into cobble-stone by day 21, indicating differentiation into osteoblasts. This is in accordance to the measurement of ALP (Fig. 8). An increase of the ALP activity is seen on the first day, which indicates the onset of osteogenesis[15]. For the polymer and TCP samples the values did not change significantly, but for the composite, at day 14, they were lower than day 1. A decrease in ALP activity is associated with cell mineralisation[15], which was confirmed for the PLLA/PGF samples through Alizarin red staining and XPS. These deposits were not found for polymer samples, despite the presence

of osteoblasts at the surface. Figure 9(a) clearly indicates that mineral leaches from the fibres as they degrade, but also that the small amount of mineral which leached from the fibres stimulated further mineralisation when cells were seeded onto the materials.

Figure 9- Alizarin red staining of cell-seeded PLLA disc, and PLLA/PGF discs in supplemented media only and with cells. a) Micrographs of samples collected at day 1, 7, 14 and 21. Red stain indicated presence of calcium at the sample surface (scale bar: 500 μm for d1 and d7, 1 mm day 14 and day 21). b) Measurement of calcium and phosphate atomic percentage at the sample surface using XPS. * representing the significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

As noted in the introduction, load bearing resorbable polymer composites reinforced with phosphate glass or hydroxyapatite have been widely studied [6,7], but where osteoinduction is investigated the materials usually give an osteoinductive effect after the polymer has

started to degrade, as only then can sufficient ion leaching occur to stimulate a noticeable effect. The use of short fibres seems to allow more material to present on the surface of the composite, allowing the osteoinductive effect to occur almost immediately. This may be as a result of the higher aspect ratio, and higher surface area for a given volume of material, of these fibres when compared to particulates. The higher aspect ratio will make it more difficult for the fibres to flow through the molten or semi-solid polymer, and the higher surface area will make it more likely that a part of the fibre will be accessible at the surface of the material.

4.3 Suitability as fixation device

Overall we consider that the PLLA/PGF would offer a similar material to the PLLA/hydroxyapatite material used in the OSTEOTRANS MX devices[16], but with the potential advantage of an enhanced osteostimulatory effect, which has potential to be of value in treating osteoporotic fractures where such a stimulus may help overcome the limited regenerative capacity of the host. The speed of dissolution of phosphate glasses in bulk can cause a cytotoxic response[8], but the low volumes accessible at the surface of the PLLA/PGF composite have shown a clear osteoinductive effect. Mechanically, the PLLA/PGF composites are initially at the lower range for cortical bone in terms of flexural strength (~50 MPa)[17], but then drop to offer average strengths in the range of 20-50 MPa over the course of 8 weeks in PBS. Such values would allow for the use of these materials in small fracture, mainly in areas where smaller loads are applied, such as maxilla and craniofacial fractures[18]. The retention of mechanical properties for up to 8 weeks in PBS suggests the material will offer support for the 35 day period typically needed for bone healing[19]. For a bone fixation application the need is for bone growth to be stimulated in the vicinity of the implant rather than on and around the implant, and so device design will need to take into account the need to stimulate healing without creating significant unhelpful

bone growth. The long term dissolution behaviour and possible device designs will now be studied to ensure that the material is appropriate for long term in vivo application, and that devices capitalise effectively on the material osteoinductivity.

5. Conclusions

This paper presents a new biocomposite material, PLLA reinforced with short PGFs, and demonstrates that the presence of the PGFs provides a clear and immediate osteoinductive effect over a period of three weeks, without compromising the mechanical properties over a period of 8 weeks in vitro testing. The osteoinduction arises from dissolution of the PGF fibres available at the surface of the composite, which is considered to be enhanced by the aspect ratio of the short fibres, with the mineral released by the fibres then stimulating cells seeded onto the material to further mineralise. Overall, the developed composite material has promise in fracture fixation applications. The ability to stimulate bone formation could accelerate the process of bone repair, improving patient recovery, particularly for patients with limited bone regeneration capability.

6. Acknowledgements

The research was funded in part by the EPSRC Centre for Doctoral Training in Additive Manufacturing and 3D Printing (EP/L01534X/1), the EPSRC Centre for Innovative Manufacture in Medical Devices (EP/K029592/1), and Glass Technology Services Ltd., Sheffield, UK.

References

- [1] C.S. Colón-Emeric, K.G. Saag, Osteoporotic fractures in older adults, *Best Pract. Res. Clin. Rheumatol.* 20 (2006) 695–706. doi:10.1016/j.berh.2006.04.004.
- [2] P. Giannoudis, S. Psarakis, G. Kontakis, Can we accelerate fracture healing?. A critical

- analysis of the literature, *Injury*. 38 (2007). doi:10.1016/j.injury.2007.02.013.
- [3] M.S. Taljanovic, M.D. Jones, J.T. Ruth, J.B. Benjamin, J.E. Sheppard, T.B. Hunter, Fracture fixation, *Radiographics*. 23 (2003) 1569–1590. doi:10.1148/rg.236035159.
 - [4] T.A. Einhorn, L.C. Gerstenfeld, Fracture healing: mechanisms and interventions, *Nat Rev Rheumatol*. 11 (2015) 45–54. <http://dx.doi.org/10.1038/nrrheum.2014.164>.
 - [5] R.R.M. Bos, G. Boering, F.R. Rozema, J.W. Leenslag, Resorbable poly (L-lactide) plates and screws for the fixation of zygomatic fractures, *J. Oral. Maxillofac. Surg.* 45 (1987) 751–753.
 - [6] G. Tripathi, P. Choudhury, B. Basu, Development of polymer based biocomposites: a review, *Mater. Technol.* 25 (2010) 158–176. doi:10.1179/175355510X12723642365089.
 - [7] N. Ramesh, S.C. Moratti, G.J. Dias, Hydroxyapatite–polymer biocomposites for bone regeneration: A review of current trends, *J. Biomed. Mater. Res. Part B Appl. Biomater.* 106 (2017) 2046–2057. doi:10.1002/jbm.b.33950.
 - [8] R.M. Felfel, I. Ahmed, A.J. Parsons, G. Palmer, V. Sottile, C.D. Rudd, Cytocompatibility, degradation, mechanical property retention and ion release profiles for phosphate glass fibre reinforced composite rods, *Mater. Sci. Eng. C*. 33 (2013) 1914–1924. doi:10.1016/j.msec.2012.12.089.
 - [9] R.K. Roeder, G.L. Converse, R.J. Kane, W. Yue, Hydroxyapatite-Reinforced Polymer Biocomposites for Synthetic Bone Substitutes, (2008).
 - [10] T. Swift, R. Hoskins, R. Telford, R. Plenderleith, D. Pownall, S. Rimmer, Analysis using size exclusion chromatography of poly(N-isopropyl acrylamide) using methanol as an eluent, *J. Chromatogr. A*. 1508 (2017) 16–23. doi:<https://doi.org/10.1016/j.chroma.2017.05.050>.
 - [11] I.B. Rabinovich, A.A. Labashov, V.I. Kucheryavyi, No Title, *Russ. J. Phys. Chem.* 34 (1960).
 - [12] S. James, J. Fox, F. Afsari, J. Lee, S. Clough, C. Knight, J. Ashmore, P. Ashton, O. Preham, M. Hoogduijn, R. De Almeida, R. Ponzoni, Y. Hancock, M. Coles, P. Genever, Multiparameter Analysis of Human Bone Marrow Stromal Cells Identifies Distinct Immunomodulatory and Differentiation-Competent Subtypes, *Stem Cell Reports*. 4 (2015) 1004–1015. doi:10.1016/j.stemcr.2015.05.005.
 - [13] M. Cocca, M.L. Di Lorenzo, M. Malinconico, V. Frezza, Influence of crystal polymorphism on mechanical and barrier properties of poly(l-lactic acid), *Eur. Polym. J.* 47 (2011) 1073–1080. doi:<https://doi.org/10.1016/j.eurpolymj.2011.02.009>.
 - [14] V. Krishnan, R. Dhurjati, E.A. Vogler, A.M. Mastro, Osteogenesis in vitro: from pre-osteoblasts to osteocytes: a contribution from the Osteobiology Research Group, The Pennsylvania State University., *In Vitro Cell. Dev. Biol. Anim.* 46 (2010) 28–35. doi:10.1007/s11626-009-9238-x.
 - [15] G.S. Stein, J.B. Lian, T.A. Owen, Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation., *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* 4 (1990) 3111–3123.

- [16] S. Sukegawa, T. Kanno, N. Katase, A. Shibata, Y. Takahashi, Clinical Evaluation of an Unsintered Hydroxyapatite / Poly-L-Lactide Osteoconductive Composite Device for the Internal Fixation of Maxillofacial Fractures, *J. Craniofac. Surg.* 27 (2016) 1391–1397. doi:10.1097/SCS.0000000000002828.
- [17] L.L. Hench, Bioceramics: From Concept to Clinic, *J. Am. Ceram. Soc.* 74 (1991) 1487–1510. doi:10.1111/j.1151-2916.1991.tb07132.x.
- [18] M. Navarro, A. Michiardi, O. Castan, J.A. Planell, Biomaterials in orthopaedics, (2008) 1137–1158. doi:10.1098/rsif.2008.0151.
- [19] R. Marsell, T.A. Einhorn, The biology of fracture healing, 42 (2012) 551–555. doi:10.1016/j.injury.2011.03.031.THE.