Cytocompatibility, degradation, mechanical property retention and ion release profiles for phosphate glass fibre reinforced composite rods

R. M. Felfel,[1,2*] I. Ahmed,[1] A. J. Parsons, [1], G. Palmer, [3] V. Sottile, [4] and C. D. Rudd [1]

[1] *Faculty of Engineering, Division of Materials, Mechanics and Structures, University of Nottingham, Nottingham NG7 2RD, United Kingdom*

[2]*Physics Department, Faculty of Science, Mansoura University, Mansoura 35516, Egypt*

[3]*Biomaterials and Tissue Engineering, Eastman Dental Institute, University College London, 256 Gray's Inn Road, London, WC1X 8LD, United Kingdom*

[4]*Wolfson Centre for Stem Cells, Tissue Engineering and Modelling (STEM), School of Clinical Sciences; Centre for Biomolecular Sciences, University of Nottingham, Nottingham NG7 2RD, United Kingdom*

[*]*Email: eaxrf1@nottingham.ac.uk*

Abstract:

Fibre reinforced composites have recently received much attention as potential bone fracture fixation applications. Bioresorbable composites based on poly lactic acid (PLA) and phosphate based glass fibre were investigated according to ion release, degradation, biocompatibility and mechanical retention profiles. The phosphate based glass fibres used in this study had the composition of $40P_2O_5$ - 24Mg O - 16CaO - $16Na_2O$ - $4Fe_2O_3$ in mol% (P40). The degradation and ion release profiles for the composites showed similar trends with the amount of sodium and orthophosphate ions released being greater than the other cations and anions investigated. This

was attributed to low Dietzal's field strength for the Na^+ in comparison with Mg^{2+} and Ca^{2+} and breakdown of longer chain polyphosphates into orthophosphate ions. P40 composites exhibited good biocompatibility to human mesenchymal stem cells (MSCs), which was suggested to be due to the low degradation rate of P40 fibres. After 63 days immersion in PBS at 37 °C, the P40 composite rods lost ~ 1.1 % of mass. The wet flexural, shear and compressive strengths for P40 UD rods were ~ 70 %, ~ 80 % and ~ 50 % of their initial dry values after 3 days of degradation, whereas the flexural modulus, shear and compressive strengths were ~ 70 %, ~ 80 %, and ~ 65 % respectively. Subsequently, the mechanical properties remained stable for the duration of the study at 63 days. The initial decrease in mechanical properties was attributed to a combination of the plasticisation effect of water and degradation of the fibre-matrix interface, with the subsequent linear behaviour being attributed to the chemical durability of P40 fibres. P40 composite rods showed low degradation and ion release rates, good biocompatibility and maintained mechanical properties similar to cortical bone for the duration of the study. Therefore, P40 composite rods have huge potential as resorbable intramedullary nails or rods.

Keywords:

Bioresorbable – Phopshate glass fibres – Mesenchymal Stem Cell - Composite rods – Ion relaease - Cytocompatibility

Introduction:

Intramedullary (IM) nailing has been used for fracture fixation in long bones such as the femur since the 1940s [1]. IM nails or rods are commonly inserted into the medullary cavity and locked distally and proximally with screws to provide rotational and compressive stability for the fracture. Commercial IM nails are usually made of metals such as stainless steel and titanium [2].

However, complications such as implant removal, stress shielding, corrosion and risk of re-fracture have driven researchers within the biomaterials field to seek alternative materials [2-6].

Poly lactic acid (PLA) is one of the most commonly used polymers in biomedical applications. The degradation time for PLA can be tailored via copolymerisation with other bioresorbable polymers such poly glycolic acid (PGA for e.g.) depending on the end application. The mechanical properties for PLA and copolymers alone are inadequate for high stress (load-bearing) applications such as bone fracture fixation and several attempts have been made to improve their mechanical performance. A self-reinforcement (SR) technique developed by Tormala *et al.* [7, 8] based on hot drawing of polymers below the melt temperature or hot compaction of polymer fibres, was applied to polymers such as PLA and PGA. High mechanical strength composites were produced, however, their stiffness or modulus did not increase by the same percentage as their strength. The flexural strength and modulus for these SR-PLA rods ranged from 160 to 270 MPa and from 3.5 to 16 GPa respectively, [9-13] depending on the chemical composition of the PLA and draw ratio. Their shear and compressive strength were recorded at being ~ 100 MPa and ~ 80 MPa respectively [12, 13]. A further drawback with the use of pure PLLA or PGA devices is reports of late inflammation in *in vivo* studies due to acidic degradation products [14-17]. Thus, alternative implant materials such as composites are required to overcome these limitations.

Particulate reinforced composites have also been examined and the one investigated most commonly is PLA reinforced with hydroxyapatite (HA) or other types of calcium phosphate (CaP). PLLA rods reinforced with 40% HA were found to have values of ~ 270 MPa flexural strength and ~ 10 GPa modulus. The flexural strength and modulus values for pure PLLA are ~ 260 MPa and ~ 6.5 GPa respectively [18-21]. Biocompatibility for these rods (PLLA/HA) was

examined via *in vivo* experiments using a rabbit femur model for 52 weeks [20]. The rods were implanted into the medullary canals of the femurs through their knees. No inflammatory reaction was reported for any time point of the experiment. The flexural properties for particulate composite rods based on PLA and 25 % β-calcium metaphosphate were recorded to be 120 MPa (for strength) and 4.3 GPa (for modulus) [22]. The mechanical properties for the self-reinforced composites mentioned above were much higher in comparison to these particulate reinforced composites.

Attempts have also been made to combine the advantages of both of the previous two methods, i.e. using self-reinforcement in addition to also incorporating osteoconductive particles such as bioactive glass 13-93 (BaG) in order to accelerate bone healing [13, 23, 24]. Niemela *et al.* [13] investigated the initial mechanical properties for SR-PLA rods reinforced with 0 % to 50 % of BaG particles. They found the initial flexural, shear and compressive strength decreased gradually with increasing BaG content. They ascribed this reduction in mechanical properties to the formation of pores, discontinuity of the structure and the absence of chemical bonds between BaG particles and PLA. However, improvement in bioactivity was reported for these BaG particle reinforced specimens as precipitation of calcium phosphate was seen on the composite surface after 4 days of immersion in PBS at 37^{o}C.

Fibre reinforcement is another common method employed to enhance strength and stiffness of polymers, although this approach is uncommon with degradable systems due to limitations in appropriate reinforcement materials. Phosphate based glass fibres (PGF) have been investigated for this purpose as these glasses have unique advantages such as degradability, biocompatibility and bioactivity [25-29]. The degradation rate for PGF can be extended easily from days to months or even years just by altering their chemical composition [30-35]. Biocompatibility for

fibre reinforced composites (PCL/PBG and PCL/45S5 Bioglass) was examined by Scotchford *et al.* [36] via implantation in the skulls of rats. No inflammation was seen for either composite for the duration of the study at 26 weeks. Moreover, new bone formed below the composite implant, which was confirmed using micro-computed tomography (μ-CT) [28, 37]. This new bone growth was not observed with the control samples and so it was surmised that PBG are capable of promoting new bone formation.

The authors had previously investigated degradation and mechanical retention of bioresorbable fibre reinforced composite rods based on PLA and PGFs of $50P_2O_5$-40CaO-$5Na_2O$-$5Fe_2O_3$ (in mol% - P50), however, they found that these fibres degraded far too quickly for application as a bone fracture fixation device [38]. Thus, in the present study durable glass fibres ($40P_2O_5$-24MgO-16CaO-$16Na_2O$-$4Fe_2O_3$ in mol%) [39, 40] were used to produce unidirectional (UD), chopped strand random fibre mats (RM) and a mixture of UD and RM (UD/RM) composite rods. Glass of a similar composition to that in the present study was investigated previously and showed a low degradation rate and good cytocompatibility [40, 41]. Based on the previous work on P50 composite rods (RM and UD) [38], it was concluded that the mechanical properties of RM composites were not greatly improved in comparison to PLA alone. It was recommended that UD composites be investigated in future studies. Therefore, the mechanical, degradation and ion release properties for P40 UD composite rods have been investigated in the current study.

PLA/PGF composites have also been investigated for their potential application not only for intramedullary nailing but also as bone plates and screws (refs). Moreover, Han *et al.* [42] demonstrated that drilling screw-holes into UD composite plates caused extensive damage (i.e. cracks and delamination) around the screw-holes. They found that the damage was eliminated when using RM or RM and UD combinations (UD/RM composite) instead of pure UD

composites. Thus, the biocompatibility tests in the present study were conducted on composites with different fibre architecture (RM, UD and UD/RM) to assess the effect of fibre distribution on the biocompatibility of the composites.

In the present study, ion release rates and cytocompatibility for the P40 composite rods were investigated and correlated with degradation profiles. Since these composite rods have potential applications as IM nails, Human mesenchymal stem cells (MSCs) were used for cytocompatibility testing. Furthermore, Mechanical property retention (flexural, shear and compression) of the rods was determined during the course of degradation.

1.1 Materials and Methods:

Composite Production:

Phosphate based glass fibres and mats were prepared as reported previously by Felfel *et al*. [38, 39]. Briefly, the fibres used in the current study had the composition $40P_2O_5$ - 24MgO - 16CaO - $16Na_2O$ - $4Fe_2O_3$ in mol% - denoted as P40. Continuous fibres with ~15 mm diameter were produced via melt-draw spinning at ~1100 ^oC and ~1600 rpm. The fibres were annealed for 90 minutes at the glass transition temperature T_g (T_g = 479 ^oC) prior to use.

Random non-woven fibre mats (RM) were prepared from 10 mm chopped fibres dispersed in Cellosize (Univar Ltd.). The unidirectional (UD) fibre mats were produced from continuous fibres cut into 110 mm bundles and aligned together (manually) to which Cellosize solution was gently added using a syringe, in order to bind the fibres together. The RM and UD fibre mats produced were rinsed with deionised water to remove any residual binder before being dried to constant temperature at 50 ^oC for 30 min. Composites were prepared using two different fibre architectures, UD, RM and a mixture of UD and RM (UD/RM) via a film stacking process. The

PLA films (Resin 3251-D NatureWorks® average Mw ~90,000-120,000) were stacked alternately with UDs and RMs (for UD/RM mix composites) in a 110 mm (width) x 110 mm (length) x 4.5 mm (thickness) mould placed between two metallic plates. This stack was then heated in the press for 15 mins at 210 °C and pressed for 15 mins at 38 bar. The plates were transferred to a second press for cooling to room temperature at 38 bar for 15 mins.

Composite Rod Production:

P40 UD, P40 RM and P50 UD/RM composite rods were prepared by forging at ~ 100 ºC using a specially made mould [38]. The composite laminates and pure PLA plates were cut into pieces of 100 mm length x 4.5 mm width x 3.5mm height using a band saw and were then placed into the cavity of the mould. This mould was then placed into an oven at 210 ºC and left for 10 mins. Studies showed that 10 mins were sufficient to heat up the specimen to ~ 100 ºC. After heating, the mould was transferred to a cold press, formed into shape and then cooled for 5 mins at 15 bar. Continuous unidirectional (UD) and in plane chopped strand random mat (RM) fibres within the composite rods were parallel to their longitudinal axis. The fibre volume and mass fractions of the composite rods were obtained using the matrix burn off method according to the ASTM standard test method (ASTM D2584-94) [43]. See Table 1 for details of the rods produced in this study with their respective sample codes.

Cumulative Ion Release Study:

PLA and P40 UD rods (6 mm diameter) were prepared and cut into discs of 2 mm height. These samples were degraded in 25 ml of deionised water for 1, 2, 3, 8, 10, 14, 17, 20, 24, 28 days, which was then analysed using ion chromatography for cation and anion release.

Cation Release:

An ICS-1000 ion chromatography system (Dionex, UK) was used for measurements of cation release. Na^{+}, Ca^{2+} and Mg^{2+} cations can be detected simultaneously by using this technique. A 20 mM Methanesulfonic acid (BDH, UK) solution was used as the eluent. In this method, cations were eluted using 4 x 250 mm IonPac(r) CS12A separator columns, a 25-mL sample loop, and a Cation Self-Regenerating Suppressor (CSRS). All results were calculated against a seven-point calibration curve using the predefined calibration routine. Calibration solutions were prepared from a six-cation standard stock solutions (Dionex, UK). The following standard dilutions were prepared: 200, 100, 50, 25, 12.5, 6.25, and 3.125 ppm. Data analysis was performed using the Chromeleon(r) software package (version 6.0).

Anion Release:

The phosphate anion measurements were obtained using a Dionex ICS-2500 ion chromatography system (Dionex, UK), consisting of a gradient pump with a 25-mL sample loop. In this method, polyphosphates were eluted using 4 x 250 mm IonPac(r) AS16 anion exchange columns packed with anion exchange resin. A Dionex ASRS(r) (Anion Self-Regenerating Suppressor) was used at 223 mA. The Dionex EG40 eluent generator equipped with a KOH (potassium hydroxide) cartridge was used in conjunction with the ASRS(r). The sample run time was set for 20 min. The gradient program started from 30 mM KOH, and after 2 min increased from 30 to 60 mM KOH over 11 ½ min, and remained there for 2 min. After this, it decreased down to 30 mM for 2 min. The Chromeleon(r) software package was used for data analysis. Only four reagents were commercially available for the preparation of standards - sodium phosphate tribasic (Na_3PO_4), trisodium trimetaphosphate ($Na_3P_3O_9$), pentasodium tripolyphosphate ($Na_5P_3O_{10}$), (Sigma, UK) and tetrasodium pyrophosphate ($Na_4P_2O_7$) (BDH, UK). A 100 ppm working solution containing all of the above four reagents was prepared, from which serially diluted 50, 20, 10, and 5 ppm

standard solutions were obtained. Higher phosphate group containing reagents (i.e. P4 or above) were not commercially available.

Biological assessment:

In order to evaluate the cytocompatibility of the composite samples and their effect on cell differentiation, GFP-labelled human marrow-derived mesenchymal stem cells (hMSCs) were seeded onto discs cut from the composite rods and cultured for up to 3 weeks. PLA, P40 RM, P40 UD and P40 UD/RM (70/30) composite discs of 2 mm thickness and 6 mm diameter were used for biological assessment. The cells were seeded onto polished cross sections of the composite discs with roughness coefficients of approximately 2 μm.

Cell culture:

Human mesenchymal stem cells (MSCs) labelled with green fluorescent protein (GFP) were grown as previously described [44, 45]. Briefly, cells were maintained in control medium (CTRL) containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1 mM L-glutamine, 1% nonessential amino acids and antibiotics (Invitrogen, Paisley, UK). Before cell seeding, the composite samples were transferred to a 48-well plate, sterilised in 70% ethanol and subsequently allowed to air-dry overnight in a sterile environment. Cells were harvested with Trypsin/EDTA, pelleted and seeded in CTRL medium at 30×10^4 cells/well. Cell seeding was monitored after 48 hours using a fluorescent stereomicroscope (Leica) and the medium was changed every 3 days. After 5 days, medium was changed and cells were treated for 15 days with either CTRL medium or with osteogenic medium (OS), in order to evaluate the ability of cells to undergo osteogenic differentiation under the various experimental conditions tested [46-48]. OS medium contained control medium supplemented with 100 nM

Dexamethasone (Sigma-Aldrich, UK), 10 mM β-glycerophosphate (Sigma-Aldrich, U.K.) and 0.05 mM L-ascorbic acid-2-phosphate (Sigma-Aldrich, U.K.).

Quantification of cell metabolism and differentiation:

For cell metabolic activity measurements, samples were analysed using an Alamar blue assay (AbD Serotec, UK) according to the manufacturer's instructions. Fluorescence was measured in triplicate using a Bio-TEK FLx800 micro-plate reader at 530/25 nm excitation, 590/35 nm emission. For differentiation analysis, samples were washed with PBS and fixed in 4% (v/v) paraformaldehyde at room temperature for 15 min. Mineralisation was measured as previously described [45]. Briefly, fixed wells were incubated with a 1% Alizarin-Red S solution (prepared by mixing 1 mg Alizarin Red S powder with 100 ml H2O before filtering) for 15 min, and mineral deposition was quantified by extraction of the incorporated stain [49] before absorbance measurement using a plate reader (BioTek ELx800, Fisher Scientific, Loughborough, UK) in triplicate. Measuremets of alkaline phosphatase activity were also performed in triplicate using SIGMAFAST pNPP reagents (Sigma) according to the manufacturer's instructions. At least 5 replicates of each sample were measured and the average reported.

Degradation study:

The degradation study of the rods produced was performed according to the standard BS EN ISO 10993-13:2010 [50]. Specimens of P40 UD composite rods (40 mm length and 4 mm diameter) were placed individually into glass vials. The vials were filled with 50 ml Phosphate buffered saline (PBS) (pH =7.4 ± 0.2) solution and maintained at 37 °C. pH values were measured using microprocessor pH meter (HANA Instruments pH 211, US) which was calibrated with standard buffers of 4.0 and 7.0 pH values (Fisher Scientific, UK). At various time points the specimens were extracted and blot dried before weighing. The samples were placed back into vials

containing fresh PBS solution. At each time point, 5 replicates of each sample were measured and the average reported.

The percentage wet mass change (M_w), mass loss (M_L) and water uptake (W) were determined using the following equations;

$$M_w = \left(\frac{m - m_i}{m_i}\right) \times 100\ (\%)$$

$$M_L = \left(\frac{m_d - m_i}{m_i}\right) \times 100\ (\%)$$

$$W = \left(\frac{m - m_d}{m_d}\right) \times 100\ (\%)$$

where $\boldsymbol{m}$ is the mass of degraded sample measured at time $\boldsymbol{t}$, $\boldsymbol{m_i}$ is the initial mass of the sample and $\boldsymbol{m_d}$ is the mass of the degraded sample after drying at 50 ^{o}C for four days.

Mechanical tests:

Flexural testing:

The flexural strength and modulus properties were evaluated by flexural (three-point bend) tests using a Hounsfield Series S testing machine. The measurements were performed according to standard BS 2782-10: Method 1008B:1996 [51]. A crosshead speed of 1 mm/min and a 1 kN load cell was used. The rod diameter was measured at the mid-span using vernier callipers and the measurements were conducted on wet samples (80 mm length and 4 mm diameter) in triplicate (n = 3). At various time points the specimens were extracted from the degradation medium (PBS) at 37 ^{o}C and blot dried before testing.

Double shear test:

The shear strength and stiffness for P40 UD composite rods were measured using a modified double shear test [38] according to the standard BS 2782-3:Methods 340A and 340B:1978 [52]. The crosshead speed of the machine was 5 mm/min and the load cell capacity was 25kN. The measurements were applied on wet samples (30 mm length and 4 mm diameter) and carried out in triplicate (n = 3). At various time points the specimens were extracted from the degradation medium (PBS) at 37 ºC and blot dried before testing. The shear strength (τ) was calculated using the following equation;

$$\tau = \frac{F}{2A}$$

where F is force at fracture and A is the cross-sectional area of the test rod.

Compression Test:

The Compressive strength and stiffness values were determined using an Instron 5969 and the calculations were performed according to the standard BS ISO 3597-3: 2003 [53]. Rods were inserted vertically between two flat platens of the test machine (load was applied on the cross section). A crosshead speed of 5 mm/min and a 25 kN load cell was used. The measurements were applied on wet samples (10 mm length and 4 mm diameter) and carried out in triplicate (n = 3). At various time points the specimens were extracted from the degradation medium (PBS) at 37 ºC and blot dried before testing. The compressive strength was given by

$$\sigma = \frac{F}{A}$$

Statistical analysis:

One way ANOVA test followed by the Tukey's multiple comparison test was conducted for the results using GraphPad prism software (v. 5). Significance was determined at the 95% level of significance.

Scanning Electron Microscope (SEM):

Specimens were sputter-coated with carbon and examined using a JEOL 6400 SEM with an accelerating voltage of 10 kV in secondary electron mode (SE). SEM analysis was performed on freeze-fractured cross-sections of the composite rods.

Results:

Ion release:

Figures 1 and 2 show cations and anions released from P40 UD composites. PLA alone was used as control and showed zero ion release as expected. The total amounts of anions released were greater than the cations released. The release of both cations and anions from P40 UD composites followed an approximately linear relationship with time for the duration of the study. Sodium (Na^{+}), calcium (Ca^{2+}) and magnesium (Mg^{2+}) cations reached ~ 14, ~ 11 and ~ 6 ppm, whilst the orthophosphate ($P0_4^{3-}$), pyrophosphate ($P_2O_7^{4-}$), tripolyphosphate ($P_3O_{10}^{5-}$) and Cyclic Trimetaphospahte ($P_3O_9^{3-}$) anion levels were ~ 50, ~ 35, ~ 15 and ~ 2 ppm respectively, after 28 days of immersion of deionised water.

Biological test:

Cell seeding assessed after 48h using fluorescence stereomicroscopy showed that cells had attached to all composite samples with similar efficiency to the PLA control (see Figure 3). Further observation at day 5 highlighted that the cells had divided on the composite samples.

Comparison of composite samples revealed that the P40 RM seemed to sustain a marginally higher cell number than the other samples (PLA, P40 UD and P40 UD/RM). After 5 days, the medium was changed in the culture wells, with alternate wells receiving fresh CTRL medium or OS medium in order to assess the osteogenic response of the cells on the different composite samples. The well plates were incubated for a further 15 days in these media.

The relative cell proliferation on the composites (P40 RM, P40 UD and P40 UD/RM) was evaluated at day 20 by using the Alamar blue assay (Figure 4-a). This revealed a relatively homogeneous cellular response on the samples, with the P40 RM samples appearing to show slightly higher values in comparison to PLA, P40 UD and P40 UD/RM composites. However, the differences were not statistically significant ($P > 0.05$).

The cellular response to PLA and the different P40 composites was also assessed in terms of differentiation by measuring both the alkaline phosphatase activity (Figure 4-b) and the Alizarin red-stained mineral deposition (Figure 4-c, Figure 5) in CTRL vs OS-treated wells. No significant difference ($P > 0.05$) in cell differentiation was observed amongst the samples ((P40 RM, P40 UD and P40 UD/RM) for either medium (Figure 4-b). Results for P40UD were significantly lower ($P < 0.05$) than for the PLA, P40 RM and P40 UD/RM composites which showed comparable values ($P > 0.05$) for Alizarin red-stained assays in OS medium. On the other hand, although no significant difference ($P > 0.05$) was seen in CTRL medium between the composite specimens, these were found to be significantly greater than PLA alone ($P < 0.0001$). Composite samples appeared similarly permissive for cellular differentiation in response to OS conditions, which triggered significant up-regulation of both alkaline phosphatase activity and Alizarin red staining compared to matching controls.

Degradation study:

From Figure 6, the change in pH of PBS can be observed along with wet mass percentage for P40 UD composite rods over time at 37 ºC. Change in percentage wet mass represents the mass change of the specimens under wet conditions (i.e. after blot drying) and could be considered as the result of media uptake and mass loss effects. However, pH remained approximately stable at 7.5 during the entire degradation period of 63 days. Percentage of wet mass increased initially to ~ 0.4 % at 14 days and then decreased gradually to reach ~ - 0.4 % at the end of the study.

Percentage change in mass loss and water uptake for P40 UD rods versus degradation time can be seen in Figure 7. A gradual linear increase was seen in mass loss over time to reach ~ 1.1 % after 63 days in PBS at 37 ºC (see inset Figure 7). No statistically significant change in mass loss ($P > 0.05$) was seen for the rods between the 3 and 14 day time points. Water uptake behaved differently, initially increasing at day 7 to ~ 1% and then decreasing significantly ($P < 0.0005$) to ~0.6 % at day 14 before it stabilised ($P > 0.05$) at around 0.9 %.

Mechanical properties:

Figure 8 shows the flexural properties for P40 UD rods versus time during degradation in PBS at 37 ºC. The wet flexural strength and modulus was around 30 % less than the initial dry values (~ 240 MPa and ~ 25 GPa for strength and modulus) after 3 days of degradation. Subsequently, the strength and modulus remained at ~ 95 MPa and ~ 14 GPa until the 42 day time interval followed by a apparent decrease to 80 MPa and 10.5 GPa respectively at the final time point of the study (63 days). However, the differences in strength and modulus values between 7 and 63 days were not statistically significant ($P > 0.05$). Flexural strength and modulus between day 7 and day 63 were significantly ($P < 0.01$) lower than that at day 3.

Shear strength and stiffness for P40 UD composite rods against time are shown in Figure 9. After 3 days of immersion in PBS at 37 ºC, the shear strength and stiffness decreased by ~ 20 % in comparison to the initial results (before degradation). The shear strength decreased slightly to reach ~ 50 MPa at day 14 and remained constant ($P > 0.05$) until the final 63 day interval, whilst the stiffness fluctuated between 2.2 and 3.2 $kN.mm^{-1}$ and the change was not significant ($P > 0.05$) during the degradation period. Shear strength for the composite rods at day 3 was statistically significantly ($P < 0.01$) in comparison with the following time point (between day 7 and day 63). Shear stiffness at day 7 and day 63 was significantly ($P < 0.05$) lower than that at day 3.

Figure 10 shows the change in compressive strength and stiffness for P40 UD rods over time at 37 ºC in PBS. Before degradation, the compressive strength and stiffness were ~ 370 MPa and 22 $kN.mm^{-1}$ (respectively). The compressive strength and stiffness decreased by ~ 50 % and 35 % after 3 days compared to the initial results. Further reduction in strength and stiffness was seen at 14 days. From day 14 onwards, the compressive properties stabilised ($P > 0.05$) between ~ 100 MPa and ~ 120 MPa (for strength) and between ~ 8 $kN.mm^{-1}$ and ~ 11 $kN.mm^{-1}$ (for stiffness).

Figure 11 shows SEM micrographs of freeze fracture surfaces for P40 UD composite rods before and after different periods of degradation in PBS at 37 ºC. Before degradation, the fibres were well impregnated with the matrix (PLA) which is indicative of a good fibre/matrix interface (see Figure 11a). Short fibre pull-out and holes were observed for 14 day degraded samples (see Figure 11 b). Slightly longer fibre pull-out was seen for day 28 samples and even longer for day 63 samples, with clean fibre surfaces indicating fibre-matrix interface degradation.

Mechanical properties for rods made of PLA alone were previously published [38]. Flexural, shear and compressive strength were ~120 MPa, ~46 MPa and ~120 MPa and the flexural

modulus, shear and compressive stiffness were ~3.5 GPa, ~3.2 kN $mm^{-1}$and ~5 kN mm^{-1} respectively. No significant change was observed in the shear, compressive strength, flexural modulus and compressive stiffness for the duration of the study (63 days), whilst flexural strength and shear stiffness decreased by 30 % of the initial values at the 63 day interval.

Discussion:

Mechanical characteristics and biocompatibility are crucial in the selection of materials for bone fixation applications. Mechanical properties of the implant should match the cortical bone to ensure secure bone fracture fixation. Flexural, shear and compressive strengths for cortical bone are reported in the range ~90–180 MPa, ~43–89 MPa and ~130–210 MPa, respectively and Young's modulus ranges from 6 to 20 GPa [3, 38]. Incorporation of phosphate glass fibres (PGF) into the matrix (PLA) has been shown to improve both biological response as well as the material mechanical properties [3, 28, 38, 39, 54-56].

Ion release profiles for P40 UD rods correlated extremely well with the mass loss profiles (see Figure 12), demonstrating a linear relationship between mass loss and ion release. However, the ion release and degradation studies were conducted independently for the rods using different media (deionised water for ion release and PBS for degradation) and so such a comparison must be considered with caution. Similar findings were presented previously for PCL/PGF composites by Ahmed *et al*. [57]. Percentage of mass loss for P40 UD rods increased gradually until the end of the study at 63 days. It was difficult to correlate pH with ion release as the degradation medium was different (PBS was used for the degradation study and deionised water for ion release). Furthermore, it is well known that PBS is capable of compensating for small changes in the pH of the medium to maintain it around 7.4.

Ion release tests can be performed by either changing the medium at every time point (cumulative method) or by using a static medium (i.e. without changing the medium). Cumulative ion release was conducted in the current study in order to avoid precipitation effects [58, 59] that could have given imprecise values and incomparable results with the degradation. Lower degradation and ion release rates for P40 composites in comparison to P50 composites [38, 60, 61] were suggested to be due to their high chemical durability. The durability of the P40 composition was attributed to lower phosphate content and presence of Mg^{2+} which can enable cross-linking between phosphate chains [62]. The structure of phosphate glasses with low P_2O_5 content (40 mol% or below) have also been referred to as 'invert glasses' [32, 40, 63]. Invert phosphate glasses have low degradation rates and less acidic products in aqueous media due to a decrease in the fraction of Q^2 species [63, 64]. P50 formulations have been reported to have an infinite Q^2 chain structure which can be highly susceptible to hydration followed by hydrolysis [65]. Whereas the P40 glass composition has much shorter chains (i.e. a mixture of Q^1 's and Q^2 's) [29, 64]. These shorter chains are able to pack together tighter creating more durable glasses [62, 64]. Dissolution rates for P50 and P40 glass formulations in distilled water at 37°C were reported to be approximately 3×10^{-5} $g.cm^{-2}.h^{-1}$ and 3×10^{-6} $g.cm^{-2}.h^{-1}$ respectively [39, 41].

The content of calcium and sodium oxide within the P40 glass fibre formulation was the same (16 mol% of each). Sodium released more rapidly ($P > 0.05$) in comparison to the calcium, which was suggested to be due to the fact that Ca^{2+} ions have a stronger cross-linking effect and field strength than Na^+ [59]. The amount of Mg^{2+} ions released was lower than Ca^{2+}, even though there was more Mg in the glass. This was also attributed to the field strength effect as shown in Figure 13. The rate of cations released (determined from the gradient of the line between amount of cation released and time) decreased as the field strength of the cation increased. Dietzal's field

strength (ratio of valence (Z) to the square of ionic distance (a^2) for oxides) for Na^+, Ca^{2+}, Mg^{2+} and Fe^{3+} is 0.19, 0.33, 0.45 and 0.76 $Å^{-2}$ [66]. PO_4^{3-} anion release data were higher than that seen for the rest of the anions investigated for the P40 composites. This high amount of released PO_4^{3-} was suggested to be due to the breakdown of the longer chain polyphosphates in solution before analysis, since as PO_4^{3-} is the final breakdown product of these phosphate anion chains [57, 67].

Ca^{2+} ions play a crucial role in the chemical durability of the glass composition. This was seen by Ahmed *et al.* [29] and Bunker *et al.* [33], who observed that the degradation rate for phosphate glass decreasing as the amount of Ca^{2+} increased. Ca^{2+} ions can form a chelating structure via ionic bonds between two PO_4^{3-} anions producing strong P-O-P bonds. Fe_2O_3 content also has a significant positive effect on the durability of these glass fibres which has been attributed to formation of Fe-O-P bonds which are more hydration resistant [59]. Parsons *et al.* [68] found that the effect of metal addition on degradation rate of the phosphate glasses goes as Fe > Mg > Ca.

It was found that the dissolution rate of phosphate glasses has a direct influence on their biological performance, as their biocompatibility is enhanced when the dissolution rate decreases [35, 40, 69-73]. Ahmed *et al*. [41] showed that for an immortal muscle precursor cell line proliferation and differentiation for iron phosphate glass fibres (P_2O_5 – CaO – Na_2O) were enhanced by incorporation of iron (Fe_2O_3) due to a reduction in the solubility [41, 59]. The cell culture studies conducted here were applied to PLA and P40 composite samples and P50 fibre composites have been studied previously [74]. Cytocompatibility for composites based on PLA and annealed and non-annealed P50 fibres ($v_f \sim 13$ %) using an osteoblast cell line (MG63) for 7 days were investigated previously by Ahmed *et al*. [74]. They found that the annealed fibre reinforced composites as well as PLA alone showed higher cell viability than non-annealed fibre

composite. They ascribed this to the slower degradation rate of the annealed fibres in comparison with non-annealed fibres. Thus, it was expected that the P40 composites should have better cytocompatibility in comparison with the P50 composites [38] due to their low degradation rate.

MSC Cell differentiation on the P40 composites in this study was demonstrated by alkaline phosphatase (ALP) and the OS medium showed higher cell differentiation and mineralisation than the CTRL (see Figure 4-b, 9-c and 10). This was as expected, since the OS medium is known to promote osteogenic differentiation and mineralization in MSC cultures [46-48]. No statistically significant difference was found for cell proliferation and differentiation between the different specimens (PLA, P40 RM, P40 UD and P40 UD/RM) in CTRL and OS. Since mineralisation is an essential process for new bone formation [75], deposition of calcium phosphate on the specimens was quantified using alizarin red staining. P40 composites showed a higher deposition of calcium phosphate (CaP) in comparison with PLA when MSCs were maintained in CTRL medium (see Figure 4-c). This could suggest the ability of phosphate glass fibres (reinforcement of the composites) to stimulate new bone formation via CaP deposition. Conversely, no significant difference was found between P40 UD/RM, P40 RM and PLA for MSCs treated in OS medium, whereas P40 UD composite was lower than the rest of the samples. This may be caused by the scale of the mineral deposition achieved in response to OS conditions, which may be overshadowing any moderate differences between the composites. The saturation effect is supported by the optical images of Alizarin red staining of cell-seeded samples (see Figure 5). The stained nodules highlighting the deposited minerals (CaP) show higher density on the surface of P40 UD than seen in samples of P40 RM and P40 UD/RM composites.

Percentage wet mass change for P40 UD composite rods increased initially during the first 14 days and then decreased gradually (see Figure 6). The initial increase was due to water

absorption (water uptake) during the 7 day immersion period and the gradual decrease afterwards was suggested to be due to the gradual degradation of the fibre-matrix interface and slight degradation of the fibres. This was also the main reason for the gradual increase in mass loss after 14 days (see Figure 7). The pH of the PBS solution remained stable (~ 7.5) for the duration of the degradation study at 63 days. This was attributed to the fact that PBS can compensate for small changes in pH [39]. Fluctuation in the percentage of water uptake (PBS uptake) was suggested to be due to the variation of fibre content among the specimens at different time points.

A reduction in mechanical properties (flexural, shear and compression) was seen for P40 UD rods at 3 days of immersion in PBS (at 37 ^{o}C) in comparison to their initial properties before degradation. This reduction was suggested to be due to the plasticisation effect of water and degradation of the fibre-matrix interface. The plasticisation effect of water is temporary and can be removed by drying, whilst degradation of the fibre-matrix interface is a permanent effect [38, 39]. Wet flexural and shear strength for P40 UD rods decreased by 20 – 30 % of their initial dry strength after 3 days of immersion in PBS at 37 ^{o}C, whilst their compressive strength decreased by approximately 50 %. Inequality in the percentage of reduction amongst the mechanical properties was attributed to the influence of the fibres and fibre-matrix interface (i.e. the direction of the fibre-matrix interface compared to the direction of load applied). The interface was more significant for the compressive properties in comparison to the flexural and double shear properties as the load applied was in the same direction of the parallel fibres. The load applied during flexural and double shear tests was perpendicular to the fibre direction and therefore the interface contributed to a much lesser extent during transfer of load between the fibres and matrix. Compression failure mode was another reason; the non-degraded (dry

samples) failed either by buckling or edge failure, whilst after 3 days of immersion in PBS (i.e. wet samples), the failure modes seen were either crushing or splitting which were suggested to be due to the weak interface. [3, 38]. Failure modes were determined according to the standard BS ISO 3597-3 [53]. Rijsdijk *et al.* [76] investigated the effect of adding 10 wt% Maleic-anhydride-modified polypropylene (mPP) as a potential coupling agent on the flexural, shear and compressive properties of polypropylene (PP) reinforced with continuous unidirectional E-glass fibres. They found flexural and interlaminar shear strength had increased by ~ 30 % of their initial values, whilst the compressive strength had increased from 250 MPa to 396 MPa (~ 60 %). They concluded that the compressive strength of the composites was governed by the fibre/matrix interface (i.e. interface-dominant property). In the present study, the mechanical tests were restricted to wet specimens to acquire the actual mechanical properties of the composites under *in vitro* conditions. After 7 days of degradation, the mechanical properties remained stable at values similar to those of cortical bone for the remaining duration of the study. This was attributed to the chemical durability of the P40 fibres (see Figures 8, 9 and 10). The degradation of the fibre-matrix interface by media (PBS) can be seen from SEM micrographs of the cross-section of freeze fractured rod samples before and after degradation in PBS at 37 ºC. Before degradation, the fibres within the composites were fully covered with the PLA matrix and this was indicative of good fibre impregnation and a strong interface (see Figure 11a). After degradation, short fibre pull-out was observed and clean fibre surfaces were observed, which was attributed to the degradation of the fibre-matrix interface (see Figure 11 b, c and d).

The degradation, ion release and biocompatibility exhibited significant correlations among each other. Degradation rate and ion release studies during glass fibre breakdown can have a direct

influence on the biocompatibility of the composites. The initial reduction in mechanical properties was suggested to be mainly due to the degradation of the fibre-matrix interface. Application of coupling agents is a potential route to improving the fibre/matrix interface and maintaining the initial mechanical properties for longer periods desirable for bone fixation devices. Studies on this are underway currently, with preliminary work published [56, 77, 78].

Conclusions:

P40 composites exhibited good biocompatibility due to the chemical durability of the fibre reinforcement incorporated. Cation and anion release rates correlated well with mass loss profiles for P40 composites. The amount of ions released and mass loss increased linearly over time until the end of the study. Sodium ions were released in comparatively large quantities, whilst the orthophosphate ions were the highest released anionic species. The amount of calcium phosphate (CaP) deposition in CTRL medium was greater for the composites than PLA, although cell activity was similar. Percentage mass loss for P40 rods increased gradually to ~ 1.1 % at 63 days of degradation, whilst the water uptake varied between ~ 0.6 % and ~ 1 %. The wet flexural, shear and compressive strengths for P40 UD rods decreased by ~ 30 %, ~ 20 % and ~ 50 % of their initial dry values after 3 days. Afterwards, the composite rods maintained their mechanical properties at a similar range to that of cortical bone until the end of the study at 63 days. Based on the mechanical, ion release, degradation and biocompatibility properties, the P40 composite rods have huge potential as resorbable intramedullary nails or rods. However, further control over the fibre-matrix interface (via the use of coupling agents) would be required to help maintain the initial mechanical properties for longer periods, which would be desirable for bone fixation implants.

Acknowledgement:

The authors would like to acknowledge the financial support of the Egyptian Government.

References:

[1] Stannard JP, Bankston L, Futch LA, McGwin G, Volgas DA. Functional outcome following intramedullary nailing of the femur: a prospective randomized comparison of piriformis fossa and greater trochanteric entry portals. J Bone Joint Surg Am. 2011;93:1385-91.

[2] Lewis D, Lutton C, Wilson L, Crawford R, Goss B. Low cost polymer intramedullary nails for fracture fixation: a biomechanical study in a porcine femur model. Archives of Orthopaedic and Trauma Surgery. 2009;129:817-22.

[3] Felfel R, Ahmed I, Parsons A, Harper L, Rudd C. Initial mechanical properties of phosphate-glass fibre-reinforced rods for use as resorbable intramedullary nails. Journal of Materials Science. 2012;47:4884 - 94.

[4] Bombaci H, Gorgec M. Difficulty in removal of a femoral intramedullary nail: the geometry of the distal end of the nail. Yonsei Med J. 2003;44:1083-6.

[5] Star AM, Whittaker RP, Shuster HM, Duda J, Menkowitz E. Difficulties during removal of fluted femoral intramedullary rods. J Bone Joint Surg Am. 1989;71:341-4.

[6] Viljanen J, Pihlajamäki H, Kinnunen J, Bondestam S, Rokkanen P. Comparison of absorbable poly-L-lactide and metallic intramedullary rods in the fixation of femoral shaft osteotomies: an experimental study in rabbits. Journal of Orthopaedic Science. 2001;6:160-6.

[7] Törmälä P, Pohjonen T, Rokkanen P. Ultrahigh-strength self-reinforced polylactide composites and their surgical applications. Macromolecular Symposia. 1997;123:123-31.

[8] Törmälä P, Vasenius J, Vainionpää S, Laiho J, Pohjonen T, Rokkanen P. Ultra-high-strength absorbable self-reinforced polyglycolide (SR-PGA) composite rods for internal fixation of bone fractures: In vitro and in vivo study. Journal of Biomedical Materials Research. 1991;25:1-22.

[9] Matsusue Y, Yamamuro T, Oka M, Shikinami Y, Hyon S-H, Ikada Y. In vitro and in vivo studies on bioabsorbable ultra-high-strength poly(L-lactide) rods. Journal of Biomedical Materials Research. 1992;26:1553-67.

[10] Haltia A-M, Lähteenkorva K, Törmälä P, Helminen A, Tuominen J, Seppälä J, et al. Self-reinforcement and hydrolytic degradation of amorphous lactic acid based poly(ester-amide), and of its composite with sol-gel derived fibers. Journal of Materials Science: Materials in Medicine. 2002;13:903-9.

[11] Saikku-BÄCkstrÖM A, Tulamo RM, Pohjonen T, Törmälä P, Räihä JE, Rokkanen P. Material properties of absorbable self-reinforced fibrillated poly-96L/4 D-lactide (SR-PLA96) rods; a study in vitro and in vivo. Journal of Materials Science: Materials in Medicine. 1999;10:1-8.

[12] Majola A, Vainionpää S, Rokkanen P, Mikkola HM, Törmälä P. Absorbable self-reinforced polylactide (SR-PLA) composite rods for fracture fixation: strength and strength

retention in the bone and subcutaneous tissue of rabbits. Journal of Materials Science: Materials in Medicine. 1992;3:43-7.
[13] Niemelä T, Niiranen H, Kellomäki M, Törmälä P. Self-reinforced composites of bioabsorbable polymer and bioactive glass with different bioactive glass contents. Part I: Initial mechanical properties and bioactivity. Acta Biomaterialia. 2005;1:235-42.
[14] Gomes ME, Reis RL. Biodegradable polymers and composites in biomedical applications: from catgut to tissue engineering. Part 1 Available systems and their properties. International Materials Reviews. 2004;49:261-73.
[15] Spitalny AD. Bioabsorbable Implants. Clinics in podiatric medicine and surgery. 2006;23:673-94.
[16] Weiler A, Hoffmann RFG, Stahelin AC, Helling H-J, Sudkamp NP. Biodegradable implants in sports medicine: The biological base. Arthroscopy: The Journal of Arthroscopic & Related Surgery. 2000;16:305-21.
[17] Bergsma JE, de Bruijn WC, Rozema FR, Bos RRM, Boering G. Late degradation tissue response to poly(L-lactide) bone plates and screws. Biomaterials. 1995;16:25-31.
[18] Ashammakhi N, Gonzalez AM, Törmälä P, Jackson IT. New resorbable bone fixation. Biomaterials in craniomaxillofacial surgery: present and future. European Journal of Plastic Surgery. 2004;26:383-90.
[19] Ishii S, Tamura J, Furukawa T, Nakamura T, Matsusue Y, Shikinami Y, et al. Long-term study of high-strength hydroxyapatite/poly(L-lactide) composite rods for the internal fixation of bone fractures: a 2-4-year follow-up study in rabbits. J Biomed Mater Res B Appl Biomater. 2003;66:539-47.
[20] Furukawa T, Matsusue Y, Yasunaga T, Shikinami Y, Okuno M, Nakamura T. Biodegradation behavior of ultra-high-strength hydroxyapatite/poly (-lactide) composite rods for internal fixation of bone fractures. Biomaterials. 2000;21:889-98.
[21] Shikinami Y, Okuno M. Bioresorbable devices made of forged composites of hydroxyapatite (HA) particles and poly-L-lactide (PLLA): Part I. Basic characteristics. Biomaterials. 1999;20:859-77.
[22] Li Liao, Lin Chen, Ai-Zheng Chen, Xi-Ming Pu, Yun-Qing Kang, Ya-Dong Yao, et al. Preparation and characteristics of novel poly-L-lactide/ß-calcium metaphosphate fracture fixation composite rods. J Mater Res 2007;22:3324 - 9.
[23] Niiranen H, Pyhältö T, Rokkanen P, Kellomäki M, Törmälä P. In vitro and in vivo behavior of self-reinforced bioabsorbable polymer and self-reinforced bioabsorbable polymer/bioactive glass composites. Journal of Biomedical Materials Research Part A. 2004;69A:699-708.
[24] Niemelä T, Niiranen H, Kellomäki M. Self-reinforced composites of bioabsorbable polymer and bioactive glass with different bioactive glass contents. Part II: In vitro degradation. Acta Biomaterialia. 2008;4:156-64.
[25] Kiani A, Lakhkar NJ, Salih V, Smith ME, Hanna JV, Newport RJ, et al. Titanium-containing bioactive phosphate glasses. Philos Transact A Math Phys Eng Sci. 2012;370:1352-75.
[26] Lee YK, Choi SH. Novel Calcium Phosphate Glass for Hard-Tissue Regeneration. J Korean Acad Periodontol. 2008;28:273-98.
[27] Knowles JC, Franks K, Abrahams I. Investigation of the solubility and ion release in the glass system K2Oâ€“Na2Oâ€“CaOâ€“P2O5. Biomaterials. 2001;22:3091-6.

[28] Parsons AJ, Ahmed I, Han N, Felfel R, Rudd CD. Mimicking Bone Structure and Function with Structural Composite Materials. Journal of Bionic Engineering. 2010;7:S1-S10.
[29] Ahmed I, Lewis M, Olsen I, Knowles JC. Phosphate glasses for tissue engineering: Part 1. Processing and characterisation of a ternary-based P2O5-CaO-Na2O glass system. Biomaterials. 2004;25:491-9.
[30] Vitale-Brovarone C, Novajra G, Lousteau J, Milanese D, Raimondo S, Fornaro M. Phosphate glass fibres and their role in neuronal polarization and axonal growth direction. Acta Biomaterialia. 2012;8:1125-36.
[31] Ropp RC. Inorganic polymeric glasses: Elsevier; 1992.
[32] Brauer DS, Karpukhina N, Law RV, Hill RG. Effect of TiO2 addition on structure, solubility and crystallisation of phosphate invert glasses for biomedical applications. Journal of Non-Crystalline Solids. 2010;356:2626-33.
[33] Bunker BC, Arnold GW, Wilder JA. Phosphate glass dissolution in aqueous solutions. Journal of Non-Crystalline Solids. 1984;64:291-316.
[34] Ahmed I, Lewis MP, Knowles JC. Quantification of anions and cations from ternary phosphate based glasses with fixed 50 and 55 mol% P[2]O[5] using ion chromatography. 2005;46:6.
[35] Abou Neel E, Knowles J. Physical and biocompatibility studies of novel titanium dioxide doped phosphate-based glasses for bone tissue engineering applications. Journal of Materials Science: Materials in Medicine. 2008;19:377-86-86.
[36] Scotchford CA, Shataheri M, Chen PS, Evans M, Parsons AJ, Aitchison GA, et al. Repair of calvarial defects in rats by prefabricated, degradable, long fibre composite implants. J Biomed Mater Res A. 2010;96:230-8.
[37] Efeoglu C, Burke JL, Parsons AJ, Aitchison GA, Scotchford C, Rudd C, et al. Analysis of calvarial bone defects in rats using microcomputed tomography: potential for a novel composite material and a new quantitative measurement. British Journal of Oral and Maxillofacial Surgery. 2009;47:616-21.
[38] Felfel RM, Ahmed I, Parsons AJ, Walker GS, Rudd CD. In vitro degradation, flexural, compressive and shear properties of fully bioresorbable composite rods. Journal of the Mechanical Behavior of Biomedical Materials. 2011;4:1462 - 72.
[39] Felfel RM, Ahmed I, Parsons AJ, Haque P, Walker GS, Rudd CD. Investigation of Crystallinity, Molecular Weight Change, and Mechanical Properties of PLA/PBG Bioresorbable Composites as Bone Fracture Fixation Plates. J Biomater Appl. 2012;26:765 - 89.
[40] Ahmed I, Parsons A, Jones A, Walker G, Scotchford C, Rudd C. Cytocompatibility and Effect of Increasing MgO Content in a Range of Quaternary Invert Phosphate-based Glasses. J Biomater Appl. 2010;24:555-75.
[41] Ahmed I, Collins CA, Lewis MP, Olsen I, Knowles JC. Processing, characterisation and biocompatibility of iron-phosphate glass fibres for tissue engineering. Biomaterials. 2004;25:3223-32.
[42] Han N, Ahmed I, Parsons AJ, Harper L, Scotchford CA, Scammell BE, et al. Influence of screw holes and gamma sterilization on properties of phosphate glass fiber-reinforced composite bone plates. J Biomater Appl. 2011.
[43] ASTM D2584-94, Standard Test Method for Ignition Loss of Cured Reinforced Resins 1994.

[44] Sottile V, Thomson A, McWhir J. In vitro osteogenic differentiation of human ES cells. Cloning Stem Cells. 2003;5:149-55.
[45] Rashidi H, Strohbuecker S, Jackson L, Kalra S, Blake AJ, France L, et al. Differences in the Pattern and Regulation of Mineral Deposition in Human Cell Lines of Osteogenic and Non-Osteogenic Origin. Cells Tissues Organs. 2011.
[46] Sottile V, Halleux C, Bassilana F, Keller H, Seuwen K. Stem cell characteristics of human trabecular bone-derived cells. Bone. 2002;30:699-704.
[47] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143-7.
[48] Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem. 1997;64:295-312.
[49] Gregory CA, Gunn WG, Peister A, Prockop DJ. An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. Anal Biochem. 2004;329:77-84.
[50] BS EN ISO 10993-13:2010, Biological evaluation of medical devices. Identification and quantification of degradation products from polymeric medical devices
[51] BS 2782-10:Method 1008B:1996, ISO 3597-2:1993, Methods of testing plastics. Glass reinforced plastics. Determination of flexural strength on rods made of roving-reinforced resin
[52] BS 2782-3:Methods 340A and 340B:1978, Methods of testing plastics. Mechanical properties. Determination of shear strength of moulding material. Determination of shear strength of sheet material
[53] BS ISO 3597-3: 2003, Textile-glass-reinforced plastics. Determination of mechanical properties on rods made of roving-reinforced resin. Determination of compressive strength
[54] Navarro M, Ginebra M, Planell J, Zeppetelli S, Ambrosio L. Development and cell response of a new biodegradable composite scaffold for guided bone regeneration. Journal of Materials Science: Materials in Medicine. 2004;15:419-22.
[55] Ahmed I, Jones I, Parsons A, Bernard J, Farmer J, Scotchford C, et al. Composites for bone repair: phosphate glass fibre reinforced PLA with varying fibre architecture. Journal of Materials Science: Materials in Medicine. 2011;22:1825 - 34.
[56] Parsons AJ, Ahmed I, Haque P, Fitzpatrick B, Niazi MIK, Walker GS, et al. Phosphate Glass Fibre Composites for Bone Repair. Journal of Bionic Engineering. 2009;6:318-23.
[57] Ahmed I, Parsons AJ, Palmer G, Knowles JC, Walker GS, Rudd CD. Weight loss, ion release and initial mechanical properties of a binary calcium phosphate glass fibre/PCL composite. Acta Biomaterialia. 2008;4:1307-14.
[58] Ahmed I, Lewis MP, Nazhat SN, Knowles JC. Quantification of anion and cation release from a range of ternary phosphate-based glasses with fixed 45 mol% P2O5. J Biomater Appl. 2005;20:65-80.
[59] Abou Neel EA, Ahmed I, Blaker JJ, Bismarck A, Boccaccini AR, Lewis MP, et al. Effect of iron on the surface, degradation and ion release properties of phosphate-based glass fibres. Acta Biomaterialia. 2005;1:553-63.
[60] Alani A, Knowles JC, Chrzanowski W, Ng YL, Gulabivala K. Ion release characteristics, precipitate formation and sealing ability of a phosphate glass-polycaprolactone-based composite for use as a root canal obturation material. Dent Mater. 2009;25:400-10.

[61] Borbely P, Gulabivala K, Knowles JC. Degradation properties and ion release characteristics of Resilon and phosphate glass/polycaprolactone composites. Int Endod J. 2008;41:1093-100.
[62] Parsons AJ, Ahmed I, Rudd CD, Cuello GJ, Pellegrini E, Richard D, et al. Neutron scattering and ab initio molecular dynamics study of cross-linking in biomedical phosphate glasses. Journal of Physics: Condensed Matter. 2010;22:485403.
[63] Brow RK. Review: the structure of simple phosphate glasses. Journal of Non-Crystalline Solids. 2000;263-264:1-28.
[64] Walter G, Vogel J, Hoppe U, Hartmann P. The structure of CaO-Na2O-MgO-P2O5 invert glass. Journal of Non-Crystalline Solids. 2001;296:212-23.
[65] Abou Neel EA, Pickup DM, Valappil SP, Newport RJ, Knowles JC. Bioactive functional materials: a perspective on phosphate-based glasses. Journal of Materials Chemistry. 2009;19:690-701.
[66] Sales BC, Boatner LA, Ramey JO. Chromatographic studies of the structures of amorphous phosphates: a review. Journal of Non-Crystalline Solids. 2000;263-264:155-66.
[67] Patel A, Knowles J. Investigation of silica-iron-phosphate glasses for tissue engineering. Journal of Materials Science: Materials in Medicine. 2006;17:937-44.
[68] Parsons AJ, Burling LD, Scotchford CA, Walker GS, Rudd CD. Properties of sodium-based ternary phosphate glasses produced from readily available phosphate salts. Journal of Non-Crystalline Solids. 2006;352:5309-17.
[69] Navarro M, Ginebra MP, Planell JA. Cellular response to calcium phosphate glasses with controlled solubility. J Biomed Mater Res A. 2003;67:1009-15.
[70] Uo M, Mizuno M, Kuboki Y, Makishima A, Watari F. Properties and cytotoxicity of water soluble Na2Oâ€“CaOâ€“P2O5 glasses. Biomaterials. 1998;19:2277-84.
[71] Salih V, Franks K, James M, Hastings GW, Knowles JC, Olsen I. Development of soluble glasses for biomedical use Part II: The biological response of human osteoblast cell lines to phosphate-based soluble glasses. Journal of Materials Science: Materials in Medicine. 2000;11:615-20.
[72] Hoppe A, Güldal NS, Boccaccini AR. A review of the biological response to ionic dissolution products from bioactive glasses and glass-ceramics. Biomaterials. 2011;32:2757-74.
[73] Brauer DS, Rüssel C, Li W, Habelitz S. Effect of degradation rates of resorbable phosphate invert glasses on in vitro osteoblast proliferation. Journal of Biomedical Materials Research Part A. 2006;77A:213-9.
[74] Ahmed I, Cronin PS, Abou Neel EA, Parsons AJ, Knowles JC, Rudd CD. Retention of mechanical properties and cytocompatibility of a phosphate-based glass fiber/polylactic acid composite. Journal of Biomedical Materials Research Part B: Applied Biomaterials. 2009;89B:18-27.
[75] Maeno S, Niki Y, Matsumoto H, Morioka H, Yatabe T, Funayama A, et al. The effect of calcium ion concentration on osteoblast viability, proliferation and differentiation in monolayer and 3D culture. Biomaterials. 2005;26:4847-55.
[76] Rijsdijk HA, Contant M, Peijs AAJM. Continuous-glass-fibre-reinforced polypropylene composites: I. Influence of maleic-anhydride-modified polypropylene on mechanical properties. Composites Science and Technology. 1993;48:161-72.
[77] Haque P, Barker IA, Parsons A, Thurecht KJ, Ahmed I, Walker GS, et al. Influence of compatibilizing agent molecular structure on the mechanical properties of phosphate glass

fiber-reinforced PLA composites. Journal of Polymer Science Part A: Polymer Chemistry. 2010;48:3082-94.
[78] Haque P, Parsons AJ, Barker IA, Ahmed I, Irvine DJ, Walker GS, et al. Interfacial properties of phosphate glass fibres/PLA composites: Effect of the end functionalities of oligomeric PLA coupling agents. Composites Science and Technology. 2010;70:1854-60.

Tables and Figures:

Table 1: Sample codes for the specimens investigated in this study, with associated fibre volume and mass fractions.

Figure 1: Cumulative cations (sodium, calcium and magnesium) release versus time for P40 UD composite discs.

Figure 2: Cumulative anions (Orthophosphate ($P O_4^{3-}$), (b) Cyclic Trimetaphospahte ($P_3 O_9^{3-}$), (c) Pyrophosphate ($P_2 O_7^{4-}$) and (d) Tripolyphosphate ($P_3 O_{10}^{5-}$)) release versus time for P40 UD composite discs.

Figure 3: Cytocompatibility assessment for PLA, P40 RM, P40 UD and P40 UD/RM composite. MSC cell attachment and growth of live cells (green) were visualised under a GFP-stereomicroscope over the growing phase (2-day and 5-day time points) and after the differentiation treatment (20-day control medium and 20-day OS medium).

Figure 4: Comparison of MSC proliferation and differentiation response on different composites. (a) Alamar blue assay for CTRL medium showing the metabolic activity observed for MSC cultured on the different composites. (b) MSC differentiation analysed by alkaline phosphatase activity and (c) alizarin red staining showing the osteogenic response in CTRL and OS conditions. Alamar blue and Alkaline phosphatase provided no statistically significant

difference ($P > 0.05$) in cell proliferation and differentiation between PLA and composites in both CTRL and OS media. The alizarin red showed significant difference between the different specimens. (* is significant ($P < 0.05$) and *** is highly significant ($P < 0.0001$)).

Figure 5: Alizarin red staining of cell-seeded PLA, P40 RM, P40 UD and P40 UD/RM composites treated in (a) CTRL and (b) OS medium.

Figure 6: Change in pH and percentage of wet mass against time for P40 UD composite rods during degradation in PBS at 37 ºC.

Figure 7: Change in percentage of dry mass loss and water uptake for P40 UD composite rods versus time during degradation in PBS at 37 ºC. Inset figure shows that the mass loss followed a linear relation against degradation time.

Figure 8: Flexural strength and modulus for P40 UD composite rods versus time during degradation in PBS at 37 ºC. The initial flexural strength and modulus for dry specimens were 240 MPa and 25 GPa and results presented in this graph were conducted for wet samples.

Figure 9: Shear properties (strength and stiffness) for P40 UD composite rods against time during degradation in PBS at 37 ºC. The initial shear strength and stiffness for dry specimens were 87 MPa and 4.5 $kN.mm^{-1}$ and results presented in this graph were conducted for wet samples.

Figure 10: Change in compressive strength and stiffness for P40 UD composite rods against time during degradation in PBS at 37 ºC. The initial compressive strength and stiffness for dry specimens were 380 MPa and 22 $kN.mm^{-1}$ and results presented in this graph were conducted for wet samples.

Figure 11: SEM micrographs of a freeze fractured cross-section for P40 UD composite rods (a) before degradation, (b) after 14 days of degradation, (c) after 28 days of degradation and (d) after 63 days of degradation. Degradation study was conducted for P40 rods using PBS at 37 ºC.

Figure 12: Change in ion release at different time points against mass loss for composite rods (a) molar normalised cation release and (b) anion release. Molar normalisation was calculated via dividing of amount of released cations by the molar concentration of the cation with the glass composition (0.16, 0.16 and 0.24 for Na, Ca and Mg respectively).

Figure 13: Molar normalised rate of cation release (Na^{+}, Ca^{2+} and Mg^{2+}) versus Dietzal's field strength. Molar normalisation was calculated via dividing of rate of released cations by the molar concentration of the cation with the glass composition (0.16, 0.16 and 0.24 for Na, Ca and Mg respectively).