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Investigation of silica-iron-phosphate glasses for tissue engineering

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Abstract Phosphate-based glasses have previously been examined for tissue engineering applications [1], however they degrade rapidly in solution reducing its pH to below 5 [2]. This study presents a series of phosphate-based glass compositions that degrade at a lower rate, allowing the pH to remain close to neutral in cell culture medium. The compositions investigated were $P_{50}Ca_{30}Na_{(15-x)}Fe_5Si_x$ where x = 0, 1, 3 and 5 mol%. The dissolution and effect on pH in distilled water and cell culture medium, and ion release in distilled water were investigated over 7 days and MG63 cell attachment to glass fibres was observed after 24 hrs. Dissolution was much slower in cell culture medium (3% mass loss) compared to distilled water (50% mass loss), due to the large quantity of ions and pH buffer present. After 7 days, in cell culture medium the pH remained between 7 and 8.5, however the pH in distilled water fell to between 4 and 3, with the final pH being lower the greater the SiO_2 content. Increasing the SiO_2 content of the glass resulted in an increase in dissolution rate whilst the pH was maintained at 7 in cell culture medium. The attachment and spreading of MG63 cells was observed on all compositions. These glass compositions may therefore be suitable for tissue engineering applications.

Introduction

Tissue engineering is being investigated for the replacement/repair of damaged tissue. There is great potential for the use of degradable scaffolds for the replacement of soft and hard tissues as they would eventually be replaced by natural tissue. Materials currently being investigated for the regeneration of hard and soft tissues include synthetic polymers such as polyglycolic acid, polylactic acid and their copolymers [3, 4] and extracellular matrix components such as collagen [5] and hyaluronan [6]. However the synthetic polymers degrade catastrophically [7] and may cause inflammation and the natural polymers are mechanically weak.

Phosphate-based glasses offer a unique range of soluble materials, the degradation of which are predictable and can be controlled by altering the glass composition. Some phosphate-based glasses have previously been examined for tissue engineering applications [1, 8], however they degrade rapidly in solution reducing its pH to below 5 [2]. This would be harmful to cells cultured on these materials, it is therefore necessary to produce glass compositions that do not reduce the pH to this extent during degradation.

The incorporation of iron has been investigated in this laboratory and has shown to reduce the dissolution rate of phosphate based glasses [1]. Glasses containing 5 mol% Fe_2O_3, 50 mol% P_2O_5, 30 mol% CaO and 15 mol% Na_2O ($P_{50}Ca_{30}Na_{15}Fe_5$) have been shown to allow the adhesion and proliferation of myoblasts and the formation of myotubes *in vitro*. However, the dissolution rate of these glasses is very low (less than 0.00005 mg/cm^2/hr) [1]. Previous studies in this laboratory on ternary phosphate glass compositions have shown that the addition of SiO_2 disrupts the phosphate glass network resulting in an increase in degradation rate. It was hypothesised therefore that the addition of small quantities of silica into the $P_{50}Ca_{30}Na_{15}Fe_5$ glass composition would disrupt the phosphate network and therefore increase the dissolution rate. Anderson and Downes [9] have shown that the presence of silica allows the adhesion and proliferation of primary human osteoblasts and increases nodule formation in osteoblast cultures. Dieudonne *et al.* [10] observed a greater

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Table 1 Glass compositions investigated

Glass code	P_2O_5 mol%	CaO mol%	Na_2O mol%	Fe_2O_3 mol%	SiO_2 mol%
$P_{50}C_{30}N_{15}Fe_5$	50	30	15	5	0
$P_{50}C_{30}N_{14}Fe_5Si_1$	50	30	14	5	1
$P_{50}C_{30}N_{12}Fe_5Si_3$	50	30	12	5	3
$P_{50}C_{30}N_{10}Fe_5Si_5$	50	30	10	5	5

degree of mineralization on silica containing glass discs compared to titanium discs in addition to a higher capacity for differentiation of bone marrow stromal cells into osteoblasts.

Silicon dioxide was therefore incorporated into the composition of the glasses made in order to improve the cell adhesion properties of the materials in addition to increasing the degradation rate (see Table 1 for glass compositions investigated).

Materials and methods

Glass fibre production and characterisation

Four glass compositions were prepared using NaH_2PO_4, $CaCO_3$, P_2O_5, Fe_2O_3 and SiO_2 (Sigma-Aldrich) as starting materials. These precursors were weighed and placed into a 200 ml platinum/rhodium (Pt/10%Rh) crucible type 71040 (Johnson Matthey, Royston, UK). The crucible was placed into a furnace (Carbolite RHF 1500, UK) at between 1050 and 1100°C for 1 hr. The glass was then poured onto a steel plate and air cooled to room temperature. Fibres, of diameter 40–50 μm, were formed from the glasses via a process detailed in Ahmed *et al.* [1]. 30–50 mg of powdered glass was placed in a 100 μl Pt crucible and analysed in a Setaram differential thermal analyser (DTA), using an inert atmosphere of nitrogen and a heating rates of 20°C min^{-1} to a maximum temperature of 1000°C. The data was baseline corrected by subtracting a blank run from the plot obtained.

Fibre dissolution and effect on pH

Glass fibres of all compositions were cut into 2 cm lengths and weighed into 200 mg samples. All samples were placed into 10 ml glass vials with 10 ml of either distilled water or cell culture medium consisting of HEPES buffered Dulbecco's Modified Eagle Medium (DMEM) (Gibco, UK), supplemented with 10% foetal bovine serum (FBS) (PAA, UK) and penicillin/streptomycin (P/S) (Invitrogen, UK), and incubated at 37°C. After 1, 2, 3, 4 and 7 days the solution was removed from the samples and the remaining fibre weight was determined along with the pH of the solution. The cell culture medium was removed and replaced with fresh medium after 3 days.

Table 2 Thermal analysis data of the glass compositions investigated

Glass Code	Tg°C	Tc°C	Tm 1°C	Tm 2°C
$P_{50}C_{30}N_{15}Fe_5$	442.26	637.36	738.24	
$P_{50}C_{30}N_{14}Fe_5Si_1$	449.95	672.92	734.75	
$P_{50}C_{30}N_{12}Fe_5Si_3$	449.05	569.03	732.39	794.48
$P_{50}C_{30}N_{10}Fe_5Si_5$	469.59	698.60	792.8	

Ion release

Glass fibre samples were prepared as above in distilled water and after 1, 2, 3, 4 and 7 days 3 ml aliquots were removed and analysed by ion chromatography for Na^+, Ca^{2+} (ICS1000 Dionex, UK), Fe^{3+} and phosphate anions (ICS2500 Dionex, UK) following the methods of Ahmed I *et al.* [11].

Cell attachment

MG63 bone-like cells were seeded onto 20 mg of fibres 1 cm long of each glass composition at 5×10^5 cells per sample. The fibres were then incubated for 24 hrs at 37°C in a humid atmosphere of 5% CO_2. After incubation the samples were fixed with 3% gluteraldehyde in 0.1% sodium cacodylate buffer for 30 mins, dehydrated in a series of ethanol, then placed in hexamethyldisilozane and left to dry overnight. After drying the samples were gold coated using a Polaron E5000 sputter coater and viewed by scanning electron microscopy (SEM) (Cambridge 90B).

Results

Differential thermal analysis

The glass transition temperature (Tg) for glass code $P_{50}C_{30}N_{15}Fe_5$ was 442°C, this increased to 449°C with the substitution of 1 mol% Na_2O with SiO_2, the further substitution of 3 mol% Na_2O with SiO_2 gave a similar Tg value. However with the substitution of 5 mol% Na_2O with SiO_2 the Tg rose to 469°C. Similar trends were observed for the crystallisation (Tc) and melting (Tm) temperatures. Therefore the addition of silica in general has increased the glass transition (Tg), crystallisation (Tc) and melting (Tm) temperatures of the glass compositions.

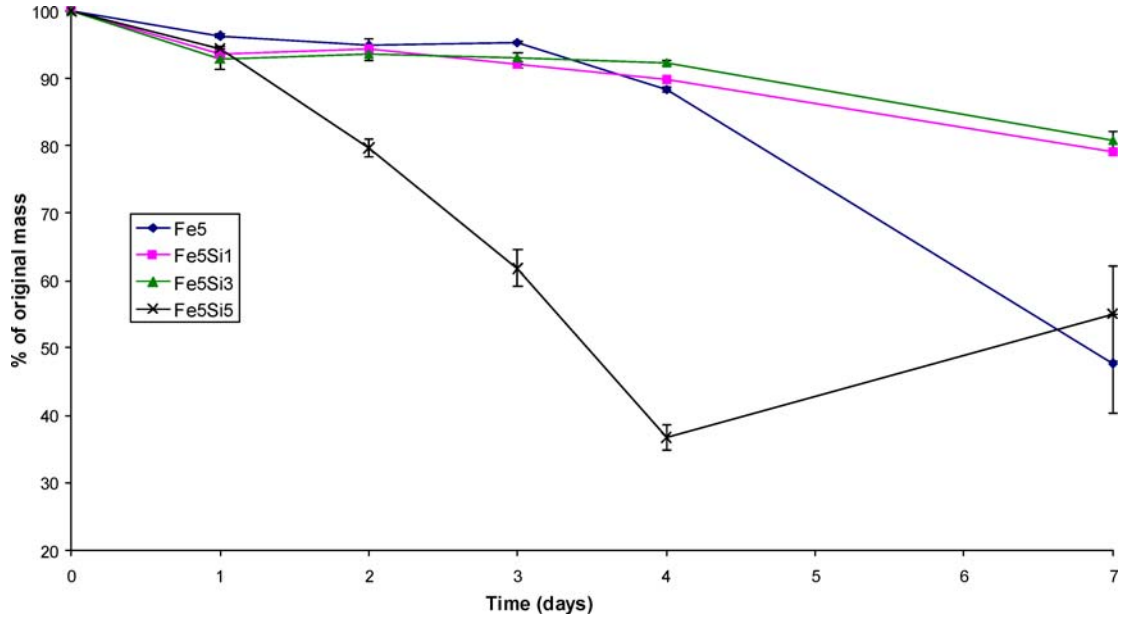

Fig. 1 Dissolution of glass fibres in water.

Glass fibre dissolution

The $P_{50}C_{30}N_{15}Fe_5$ glass fibre composition showed a 10% linear decrease in mass up to 4 days in water. After this time the glass fibres degraded at a higher rate to 45% of their original mass by 7 days (see Fig. 1). With the substitution of 1 and 3 mol% Na_2O for SiO_2 the degradation rate was significantly reduced resulting in a linear degradation rate up to day 7 and a mass loss of 20%. However, the substitution of 5 mol% Na_2O for SiO_2 resulted in the rapid non-linear degradation of the glass fibres with a mass loss of 60% at day 4. The mass of this glass composition then increased between day 4 and 7 to 55% of the original mass.

In cell culture medium the $P_{50}C_{30}N_{15}Fe_5$ glass fibre composition showed very little change in mass over the 7 days (see Fig. 2). The substitution of 1 and 3 mol% Na_2O for SiO_2 resulted in a slightly higher degradation rate which gave a mass loss of approximately 0.5% of the original mass by day 7. The most significant mass loss was observed with the substitution of 5 mol% Na_2O for SiO_2 which gave a decrease in mass of 2.5% after 7 days. The mass loss observed in water was over 20 times greater than that observed in medium.

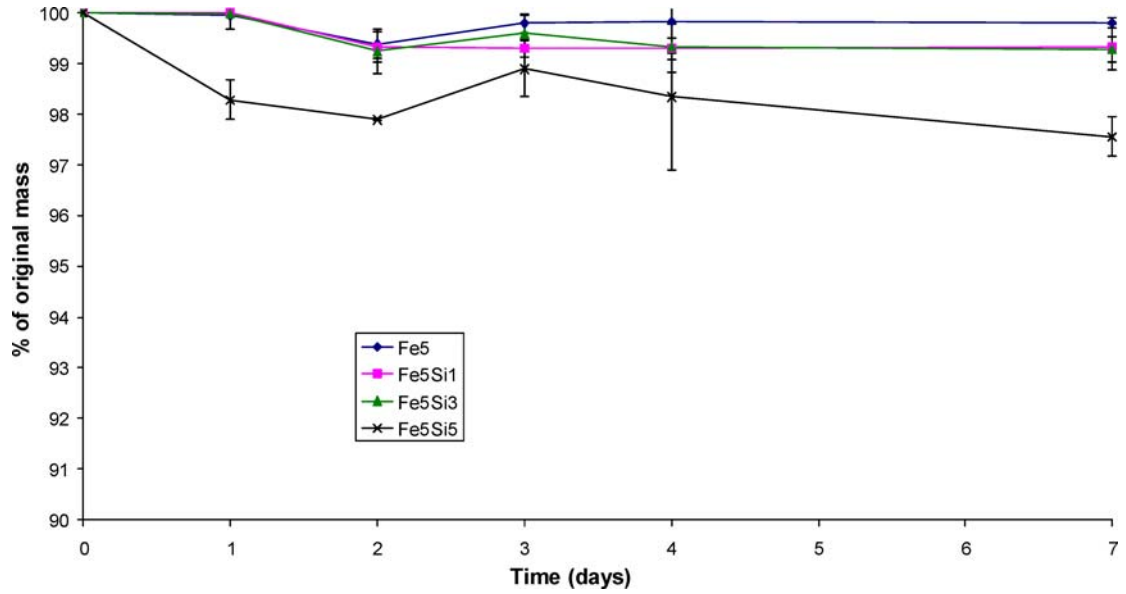

Fig. 2 Dissolution of glass fibres in cell culture medium.

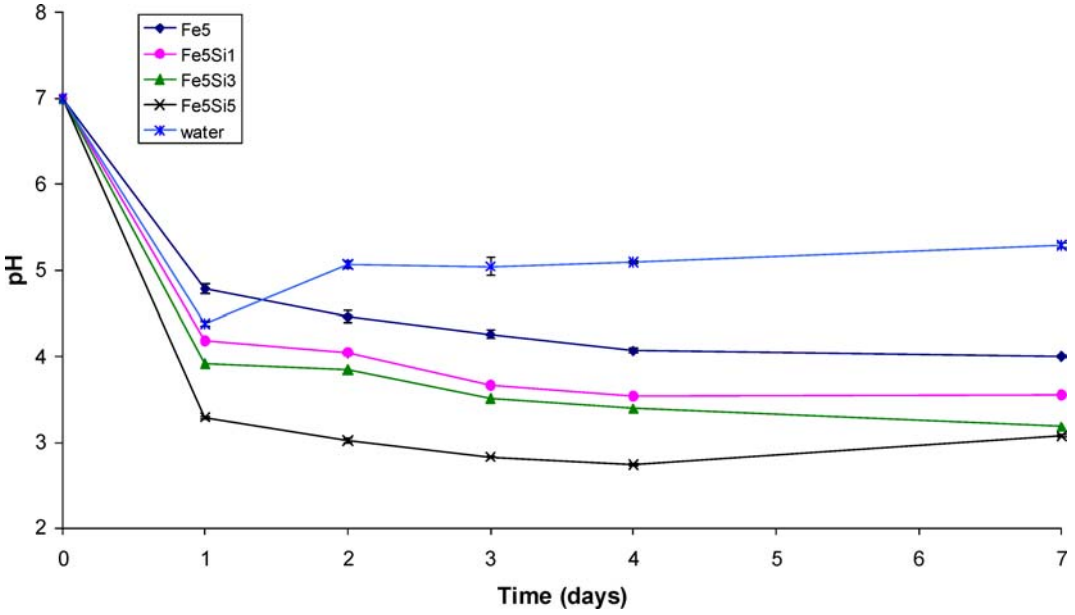

Fig. 3 The effect of glass fibres in the pH of water.

Effect of glass fibre on pH

A rapid decrease in the pH of water was observed for all compositions from day 0 to 1, which resulted in the pH of the $P_{50}C_{30}N_{15}Fe_5$ sample to fall below pH 5, that of the $P_{50}C_{30}N_{14}Fe_5Si_1$ sample to fall to pH 4, that of the $P_{50}C_{30}N_{12}Fe_5Si_3$ sample to fall just below pH 4 and that of the $P_{50}C_{30}N_{10}Fe_5Si_5$ sample to fall to just above pH 3 (see Fig. 3). From day 1–7 the pH of all the compositions decreased a further half pH except for the $P_{50}C_{30}N_{10}Fe_5Si_5$ composition, which decreased to just below pH 3 after 4 days and then increased to just above 3 after 7 days in water.

The pH of cell culture medium increased between days 0 and 3 for all compositions (see Fig. 4). The $P_{50}C_{30}N_{15}Fe_5$ and $P_{50}C_{30}N_{12}Fe_5Si_3$ compositions rose to pH 8.4 and the $P_{50}C_{30}N_{14}Fe_5Si_1$ and $P_{50}C_{30}N_{10}Fe_5Si_5$ compositions rose to pH 8.2. The pH then decreased between 3 and 4 days to between pH 7.2 and 7.8 and was then approximately stable for the remainder of the study.

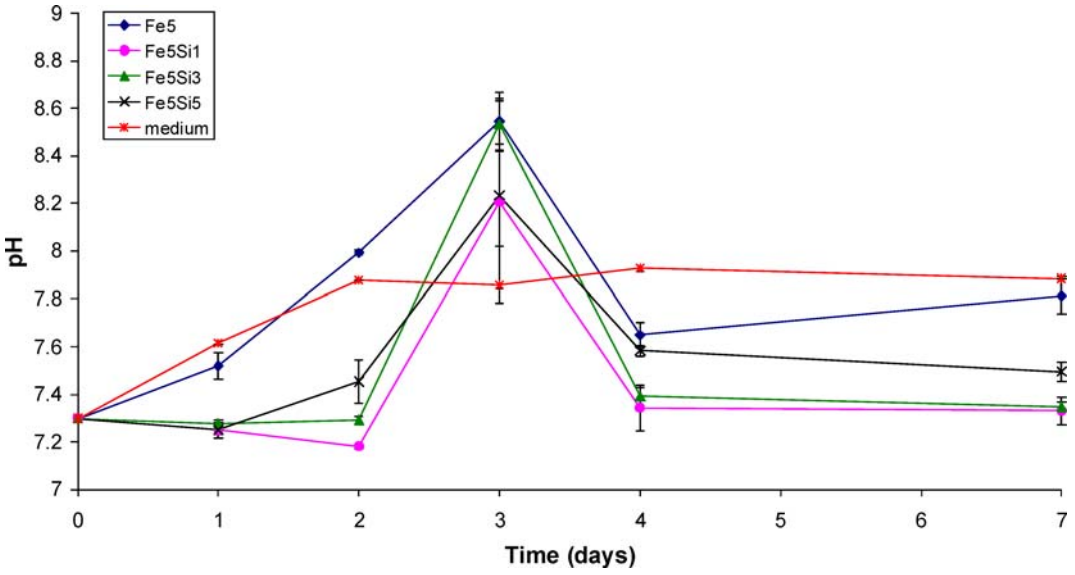

Fig. 4 The effect of glass fibres on the pH of culture medium.

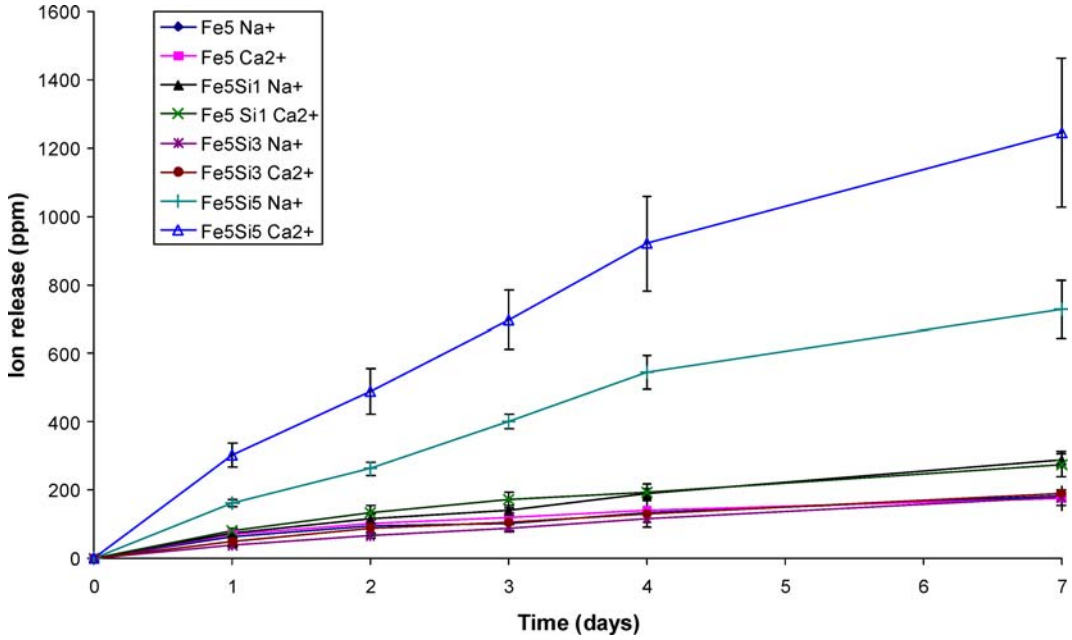

Fig. 5 Cation release from glass fibres in water.

Ion release from glass fibres

The cation release data for the $P_{50}C_{30}N_{10}Fe_5Si_5$ composition showed a rapid, linear release of ions up to day 1 followed by a slightly slower increase up to day 4 and then a slower increase in ion release to day 7 for both Ca^{2+} and Na^+ ions (Fig. 5). 1200 ppm of Ca^{2+} and 600 ppm of Na^+ was released from this composition. A linear release of ions was observed with the other compositions with 200–300 ppm of both Ca^{2+} and Na^+ ions being released. So the $P_{50}C_{30}N_{10}Fe_5Si_5$ composition released twice as many Na^+ ions and 4 times as many Ca^{2+} ions as the other compositions.

The $P_{50}C_{30}N_{10}Fe_5Si_5$ composition showed a linear increase in the quantity of PO_4 ions detected up to day 2 and then a more rapid linear observation up to day 7 (see Fig. 6). The other compositions showed a linear PO_4^{3-} ion detection profile for the entire duration of the study. Four times the quantity of PO_4^{3-} ions were detected in the $P_{50}C_{30}N_{10}Fe_5Si_5$ composition compared to the other compositions. These trends were the same for the other phosphate species

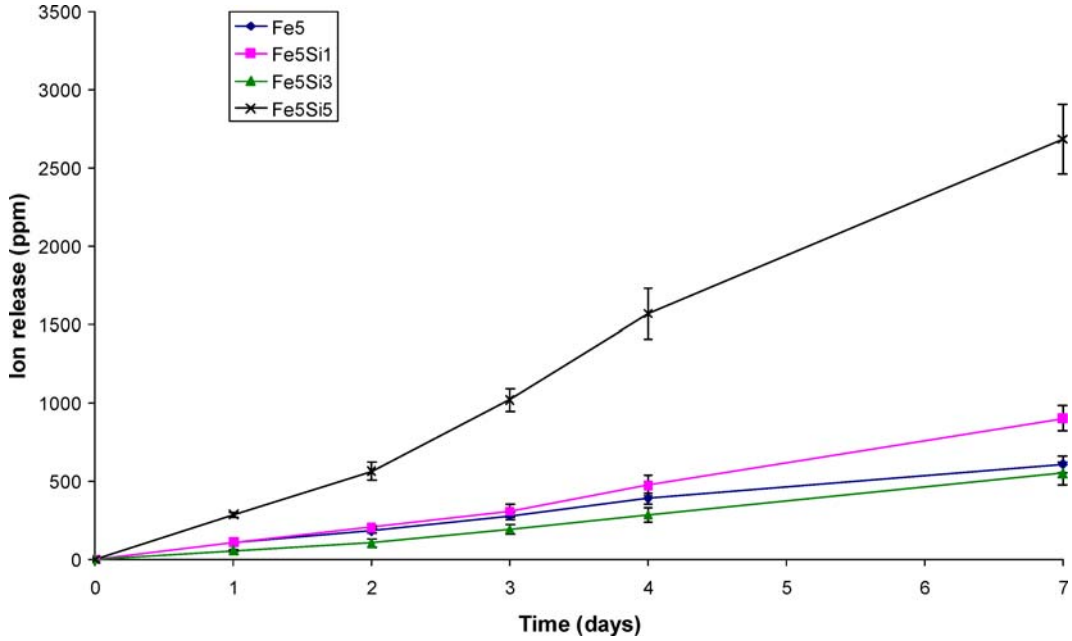

Fig. 6 PO_4 release from glass fibres in water.

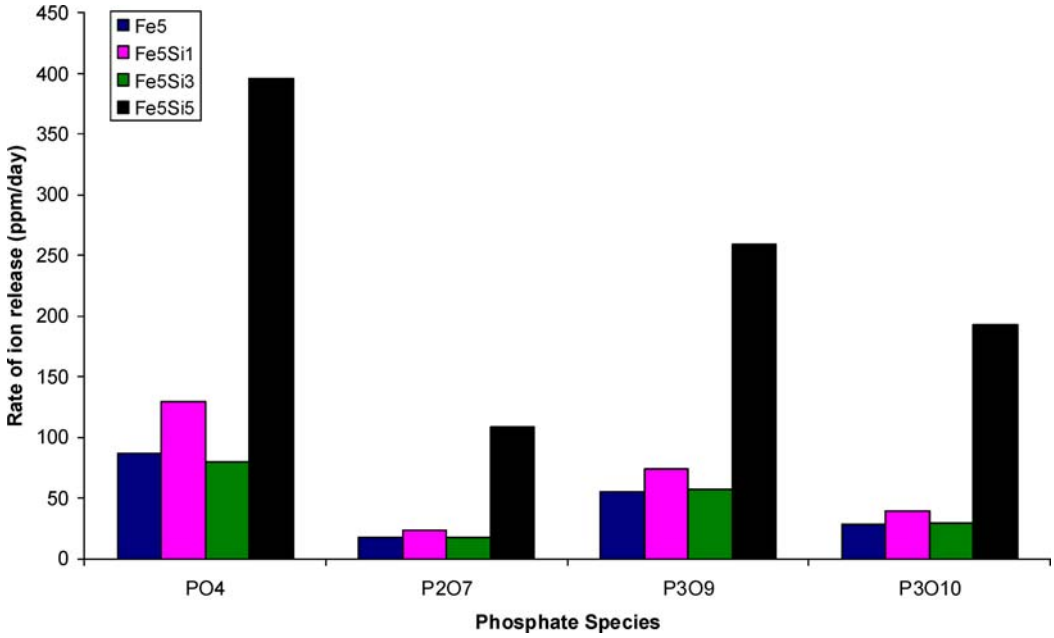

Fig. 7 Phosphate ion release from glass fibres in water.

detected, namely $P_2O_7^{4-}$, $P_3O_9^{3-}$ and $P_3O_{10}^{5-}$. The rate of ion release of all the phosphate species is summarised in Fig. 7. The rate of anion release increased with increasing SiO_2 content except for the 3 mol% SiO_2 containing composition which had an anion release rate equal to that of the $P_{50}C_{30}N_{15}Fe_5$ composition. The rate of anion release by the $P_{50}C_{30}N_{10}Fe_5Si_5$ composition was 3–4 times greater than the $P_{50}C_{30}N_{14}Fe_5Si_1$ composition. There was a greater amount of PO_4 detected than any other phosphate species.

The Fe^{3+} ion release profile was similar to the Ca^{2+} release profile for all the compositions. The quantity of Fe^{3+} ion release from the $P_{50}C_{30}N_{10}Fe_5Si_5$ composition was 3 times higher than the other 3 compositions, which showed a similar amount of release. The quantity of Fe^{3+} ions released was 3 times greater than that of Ca^{2+} ions.

Cell attachment to glass fibres

The SEM images taken after 24 hrs in culture on fibres made from the glass compositions showed that MG63 bone cells had attached to glass fibres of all compositions (Fig. 11). The cells appeared to have spread and elongated along the length of the fibre. Some preliminary quantitative experiments are underway, however problems were encountered completely removing the cells from the fibres. These experiments are still in progress.

Discussion

Quaternary phosphate glass fibres containing 1–5 mol% Fe_2O_3 were previously studied [1]. It was seen that the fibres showed a decrease in dissolution rate of an order of magnitude from 1–5 mol% Fe_2O_3. It was also seen that 4–5 mol% Fe_2O_3 was required for the attachment of muscle precursor cells [1]. Therefore the rationale for this work was to allow the modification of the glass compositions to give higher dissolution rates without producing large changes in the pH of the solution. The properties obtained have also been related to the structural changes.

With the substitution of Na_2O with SiO_2 the thermal properties tended to increase. This is expected as Na^+ additions in general tend to from non-bridging oxygens and depolymerise the structure [12]. Wallace *et.al.* [13] have also reported the observation of an increase in thermal properties with a decrease in Na_2O content.

In water the mass of all the glass fibre compositions decreased throughout the study. It was found that glass fibres with the highest silica content had the highest degradation rate. It may be that whilst the silica is a network former, the bonds formed in a SiO_2-P_2O_5 network based glass may be more sensitive to hydrolytic activity. The $P_{50}C_{30}N_{10}Fe_5Si_5$ composition degraded rapidly up to day 4 after which the mass increased to day 7. This increase in mass may have been due to the reprecipitation of released ions [14], since a white precipitate was observed in the glass vials after 7 days. The compositions containing 1 and 3 mol% SiO_2 were more stable in water than the 0 mol% SiO_2 composition. This may be due to the decrease in Na_2O in the 1 and 3 mol% SiO_2 compositions, which would lead to decrease in the proportion of non-bridging oxygens since SiO_2 is a network former and would enter the phosphate backbone of the glass.

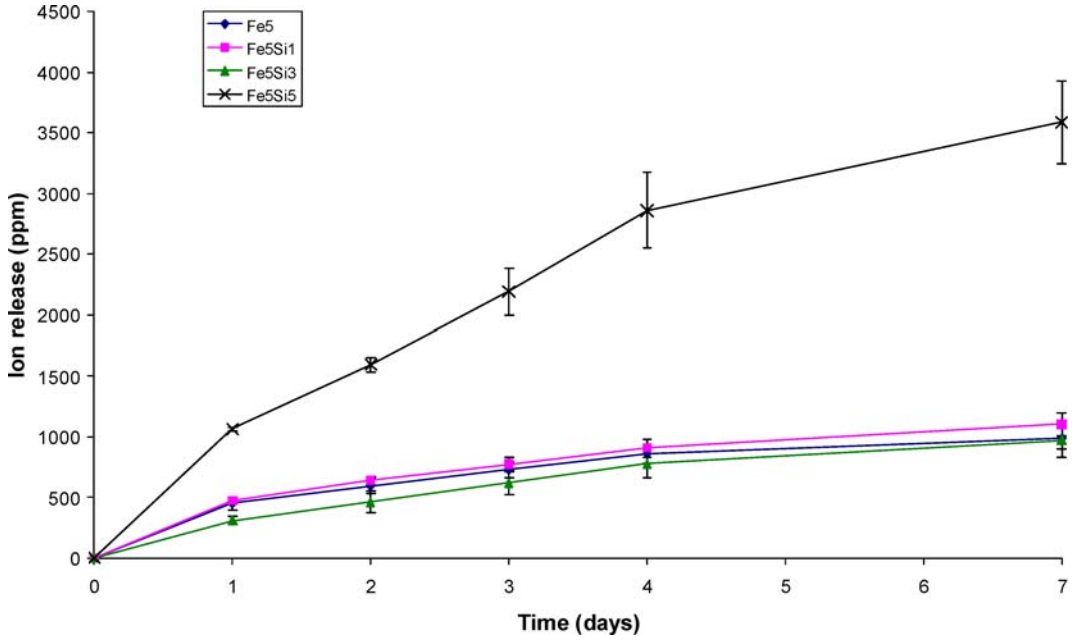

Fig. 8 Fe^{3+} release from glass fibres in water.

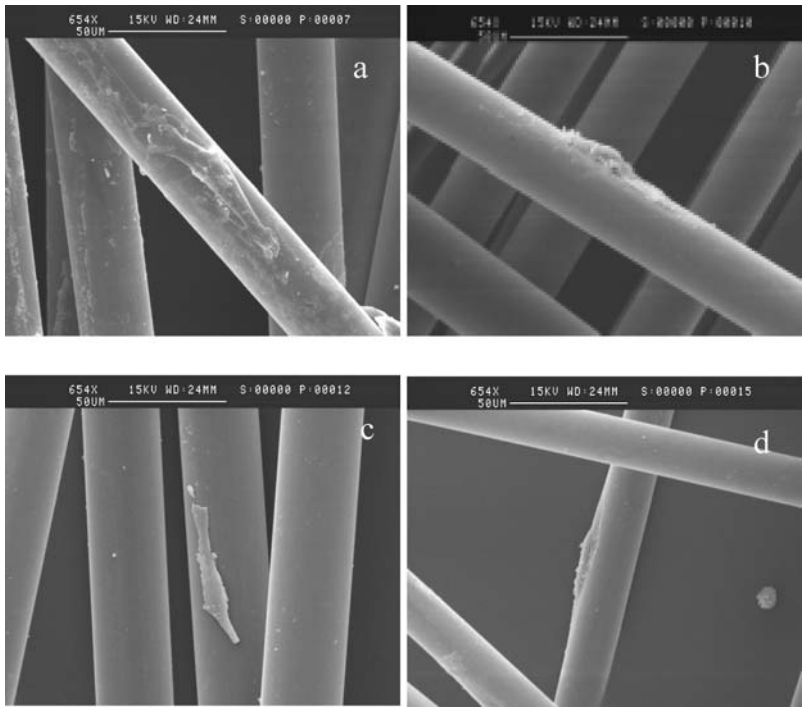

Fig. 9 MG63 osteoblast attachment to (a) $P_{50}C_{30}N_{15}Fe_5$ (b) $P_{50}C_{30}N_{14}Fe_5Si_1$ (c) $P_{50}C_{30}N_{12}Fe_5Si_3$ and (d) $P_{50}C_{30}N_{10}Fe_5Si_5$ glass fibres.

When comparing the results of the dissolution testing in water and medium, the cell culture medium consists of many large and small molecules [15] and for this reason the glass fibres may dissolve more slowly in this than water. The increase in mass between days 2 and 3 in cell culture medium may be due to the reprecipitation of ions out of solution. The medium was not changed until day 3 therefore ions were able to accumulate in the cell culture medium, which itself contains a large quantity of ions.

The pH results, in water, showed that pH decreased with increasing silica content. The pH results corroborate with the degradation and ion release data obtained, since the faster degrading composition would release the largest quantity of ions and reduce the pH to a greater degree than the other

compositions [11]. A large decrease in pH was observed for all glass compositions initially in the first day, this corroborates with the ion release data, as a high rate of ion release was observed within the first day [11]. An increase in the pH of water was observed in the $P_{50}C_{30}N_{10}Fe_5Si_5$ glass composition between days 4 and 7. This may be due to the reprecipitation of ions out of solution since it corresponds to an increase in mass and a decrease in cation release observed for this composition.

In cell culture medium the pH was seen to increase with time and then remain stable between 7 and 8.5 possibly due to the slower degradation rate of glass fibres in medium and the presence of a pH buffer within the medium. Wallace *et.al* [13] have also reported an increase in the pH of cell culture medium in the presence of P_2O_5-CaO-Na_2O glass compositions. There was an overall increase in pH up to day 3. On day 3 the medium was changed and this may explain the decrease in pH observed between days 3 and 4, since the fresh medium had a pH of 7.3. Between days 4 and 7 the pH was approximately stable.

The compositions containing 0, 1 and 3 mol% SiO_2 released approximately the same quantity of Ca^{2+}, Na^+, Fe^{3+} and phosphate ions, however the 5 mol% SiO_2 composition release 3–4 times as many. This observation is consistent with the degradation results obtained, which showed a greater mass loss for this glass fibre composition.

There was a slow initial release of phosphate ions from day 0–2, in the 5 mol% SiO_2 composition, which may have been due to the precipitation of ions out of solution since a white precipitate was observed, this may also account for the non-linearity of degradation that was also observed, a similar phenomenon was observed previously by Franks *et.al.* [14]. There was a greater concentration of PO_4^{3-} ions detected compared to the other phosphate species, possibly due to the larger phosphate species breaking down into this molecule over time. Increasing the SiO_2 content resulted in a dramatic increase in the rate of phosphate ion release from 0–5 mol% SiO_2.

Although Fe is found in both the Fe^{2+} and Fe^{3+} oxidation states in iron containing sodium phosphate glasses [16] no Fe^{2+} ion release was detected. This may be due to the oxidation of Fe^{2+} to Fe^{3+} upon contact with water. There was a greater amount of Fe^{3+} released than any of the other ions even though only 5 mol% Fe_2O_3 was added. This may be due to the reprecipitation of Ca^{2+} and phosphate ions out of solution to form a calcium phosphate. The data presented for ion release is accumulative however the daily readings showed a decrease in ion release up to day 2 followed by a slow increase to day 7 (data not shown).

MG63 bone cell attachment was observed on all the compositions after 24 hrs showing that the pH changes, degradation rate and rate of ion release did not adversely affect cell attachment. This observation is in agreement with that of Bitar *et al.* [8] who reported the attachment of MG63 cells to a glass composition of 50 mol% P_2O_5, 40 mol% CaO and 10 mol% Na_2O after 24 hrs in culture. Whilst the data presented here is qualitative, it does show the fibres will allow cell adhesion. Further experiments are planned to quantify cell proliferation and also gene expression for cells on the fibres.

Conclusion

Soluble phosphate glass compositions with a range of dissolution rates have been produced. The ion release data are in good correlation with the dissolution data and there is evidence of the reprecipitation of ions out of solution. These glass compositions do not reduce the pH of cell culture medium to the extent that it would be harmful to MG63 cells cultured on the glass. Therefore these glass compositions may be suitable materials for bone tissue engineering.

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