J Mater Sci: Mater Med (2008) 19:377–386
DOI 10.1007/s10856-007-3079-5

Physical and biocompatibility studies of novel titanium dioxide doped phosphate-based glasses for bone tissue engineering applications

E. A. Abou Neel · J. C. Knowles

Received: 18 December 2006 / Accepted: 5 April 2007 / Published online: 3 July 2007

Abstract This study investigated doping titanium dioxide (TiO_2) into phosphate glasses, 50 P_2O_5–30 CaO–20 Na_2O, to control their degradation rate and enhance their biological response to be suitable scaffolds for bone tissue engineering applications. The thermal and structural properties were analysed using differential thermal analysis and X-ray powder diffraction. The effect of TiO_2 incorporation on degradation rate, ion release, and pH changes was also carried out. In vitro cyto-biocompatibility was assessed through MG63 human osteosarcoma cells attachment and viability using scanning electron microscopy and confocal microscopy, respectively. The results showed that addition of TiO_2 produced a significant increase in density and glass transition temperature. X-ray diffraction analysis showed the presence of $NaCa(PO_3)_3$ as a main phase of these glasses with titanium phosphate Ti–P_2O_7 only detected for 5 mol% TiO_2 glasses. The degradation rate, however, was significantly reduced by one order of magnitude with incorporation of 5 mol% TiO_2 which has been reflected in released ions (cations and anions) and the minimal pH changes. Moreover, addition of TiO_2, 3 and 5 mol% in particular, supported the MG63 cells attachment and maintained high cell viability up to 7 days culture comparable to Thermanox®. These results suggested that TiO_2 containing phosphate glasses can be a promising substrate for bone tissue engineering applications.

E. A. Abou Neel · J. C. Knowles (✉)
Division of Biomaterials and Tissue Engineering,
Eastman Dental Institute, University College London,
256 Gray's Inn Road, London WC1X 8LD, UK
e-mail: j.knowles@eastman.ucl.ac.uk

Introduction

Bone loss resulting from congenital abnormalities, traumatic injury, or disease is a major health care problem worldwide. Current methods of restoring bone include transplantation of relevant tissue from healthy parts of the same patient (autograft) or from a donor (allograft or xenograft). Although the use of autograft has the best clinical outcome, it has several drawbacks such as limited donor availability, donor site infection, morbidity, pain, and additional cost of operation. On the other hand, immunological rejection of allograft or xenograft due to imperfect match can be a common problem, and lifelong immunosuppression in the recipient can cause additional morbidity and mortality [1].

To deal with these problems, tissue engineering has emerged to offer a biological substitute to the lost or damaged tissues. This depends on the use of specific cells combined with a porous biodegradable synthetic or natural scaffold, which can be formed in the shape of the defect to be replaced [2]. The scaffold should allow for cells to adhere, proliferate, and over time form a new tissue. This new tissue can be grown in vitro in the scaffold, eventually reaching an "organoid" stage where it is suitable for implantation into the patient to replace the defect, or to be allowed to grow in situ within the defect [3].

Various glasses and glass ceramics such as Bioglass®, silica based glasses which form a direct bond with the surrounding bone tissue, were clinically approved for bone applications [4–7]. Yet, there is a limitation in the compositional range that can be obtained to suit the end applications. Phosphate glasses, however, are a unique class of materials that are degradable, biocompatible, and their degradation that can be tailored to vary from days to several months. The degradation can be controlled by changing the

glass chemistry via doping the highly degradable compositions with modifying metal oxides which are known to affect the glass degradation [8–11]. Moreover, the fact that phosphate glasses contain elements that are natural constituents of the human body attract researchers interest to develop glasses for various potential soft and hard tissue engineering applications. Biocompatibility studies conducted on phosphate glasses [12, 13] showed that these glasses had no adverse effect on craniofacial osteoblasts specially those with low degradation rates [14].

Titanium is known to elicit a favourable cell response, and has been widely used as a biomaterial for several orthopaedic and dental applications [15]. In the present work, titanium dioxide (TiO_2) was incorporated into phosphate glasses to produce compositions with controlled degradation rate and enhanced biological response suitable as temporary scaffolds for bone tissue engineering applications. The thermal and structural properties of TiO_2 containing glasses were analysed using differential thermal analysis (DTA), differential scanning calorimetry (DSC), and X-ray powder diffraction (XRD). Measurement of degradation rate, ion release, and pH changes of the degrading medium were also carried out for these glasses and compared to TiO_2 free ternary glasses as controls. In vitro attachment and viability of MG63 human osteosarcoma cells cultured for 7 days on TiO_2 containing glass discs was conducted to assess the biocompatibility of these glasses and compared to both TiO_2 free ternary glasses and Thermanox® positive controls.

Experimental

Glass preparation

The precursors used in the preparation of these glasses were sodium dihydrogen orthophosphate (NaH_2PO_4), calcium carbonate ($CaCO_3$), phosphorus pentoxide (P_2O_5) and titanium dioxide (TiO_2) (BDH, Poole, UK). These were placed in a Pt/10%Rh crucible (Type 71040, Johnson Matthey, Royston, UK) which was introduced into a pre-heated furnace (Carbolite, RHF 1500, Sheffield, UK) at 700 °C to allow for removal of H_2O and CO_2 then followed by melting at the corresponding temperatures and time as given in Table 1. Upon removal of the crucible from the furnace, the melted glass was then poured into a pre-heated graphite mould at 350 and 420 °C for 1 h according to the glass composition. The mould was then allowed to cool to room temperature in the furnace overnight. Rods of 15 mm diameter for each composition were then sectioned into approximately 2 mm thick discs using a Testbourne diamond saw and methanol as a coolant.

Methods of characterisation

Density measurements

Density measurements were conducted on triplicate samples using Archimedes' Principle, on an analytical balance (Mettler Toledo, UK) with an attached density kit. Due to the soluble nature of the glass compositions investigated, ethanol was used as the submersion liquid for these measurements. The density of the glasses (ρ) were obtained employing the following equation:

$$\rho = \left(\frac{M_{\text{dry}}}{M_{\text{dry}} - M_{\text{wet}}}\right) \times \rho_{\text{liquid}}$$

where M_{dry} and M_{wet} are the masses of sample in air and liquid, respectively, and ρ_{liquid} is density of ethanol at room temperature.

Thermal analysis

Differential scanning calorimetry. Differential scanning calorimetry (DSC) was used to investigate the glass transition temperatures (T_g) of the bulk glass for all compositions using Pyris Diamond DSC (Perkin-Elmer Instruments, USA). The instrument was calibrated using the manufacturer's instructions, with indium and zinc as standards. Samples (n = 3) of 5 mg were heated, cooled and reheated from 25 to 550 °C at 100 °C min^{-1}. T_g was calculated by the onset of change in the endothermic direction (upwards) of the heat flow of the second heating ramp. All tests were carried out under nitrogen purge.

Differential thermal analysis. Differential thermal analysis (DTA) was carried out using a Setaram Differential Thermal Analyser (Setaram, France) to determine T_g, crystallisation temperature (T_c), and melting temperature (T_m). An increase in temperature from ambient up to 1,000 °C at heating rate of 20 °C min^{-1} was carried out under nitrogen purge. Samples of 60 mg were used for each test, and a blank run was carried out to baseline correct the data.

X-ray powder diffraction

X-ray powder diffraction analysis (XRD) was carried out to identify the crystalline phases present, following crystallisation of the glass. The glass compositions were crystallised at those temperatures obtained through the differential thermal analysis. The data was collected on Brüker D8 Advance Diffractometer (Brüker, UK) in flat plate geometry, using Ni filtered Cu Kα radiation. Data was collected from 10° to 100° 2θ with a step size of 0.02° and a count time of 12 s. The phases were identified using the

Table 1 Glass codes, melting and annealing temperatures used throughout this study

Glass code	P_2O_5 content (mol%)	CaO content (mol%)	Na_2O content (mol%)	TiO_2 content (mol%)	Melt temperature/ time (°C/h)	Annealing temperature/ time (°C/h)
0 mol% TiO_2	50	30	20	0	1100/1	350/1
1 mol% TiO_2	50	30	19	1	1300/3	420/1
3 mol% TiO_2	50	30	17	3	1300/3	420/1
5 mol% TiO_2	50	30	15	5	1300/3	420/1

Crystallographica Search-Match (CSM) software (Oxford Cryosystems, Oxford, UK) and the International Centre for Diffraction Data (ICDD) database (vols. 1–42).

Degradation study

The degradation studies were carried out in triplicates using a weight loss method in deionised water at 37 ± 1 °C. The surface area of the glass discs was calculated from the dimensions obtained with Mitutoyo Digimatic Vernier Callipers. Glass discs of all compositions were placed in glass bottles containing 25 mL of high purity water (18.2 MΩ cm resistivity) obtained from a PURELAB UHQ-PS (Elga Labwater, UK). The pH of this water was adjusted to 7 ± 0.1 using NH_4OH. At various time points (1, 2, 4, 24, 48, 72, 96, 168, 240 and 405 h), the solution was removed for further analysis, and the discs were dried and weighed. The discs were then placed in a fresh medium. The data was plotted as cumulative degradation, % weight loss/per unit area, as a function of time for various TiO_2 containing and free glasses.

Ion chromatography

Using ion chromatography, the degradation medium (diluted to 50×) was analysed simultaneously for cation and anion release.

Cation release measurements. The analysis of Na^+ and Ca^{2+} release was carried out using a Dionex ICS-1000 ion chromatography system (Dionex, UK). The separation was performed using 30 mM methanesulfonic acid (Fluka, UK) eluent, and a 4 × 250 mm Ion Pac® CS12A separator column and a 25-μL injection loop. Before running a sample, the ion chromatograph was calibrated against a four-point calibration curve using the predefined calibration routine. Chromeleon® software package was used for data analysis. Standard solutions were prepared using sodium chloride (Sigma, UK), and calcium chloride (BDH, UK) as reagents. A 100 ppm (parts per million) mixed (sodium and calcium) stock solution was prepared, from which serially diluted 50, 20, 10 and 1 ppm standard solutions were prepared. The column used is sensitive to phosphate species and hence samples were pre-processed through an OnGuard IIA cartridge (Dionex, UK) prior to injection on to the column.

Anion release measurements. The phosphate anion measurements were carried out using a Dionex ICS-2500 ion chromatography system (Dionex, UK), consisting of a gradient pump with a 25 μL sample loop. In this method, polyphosphates were eluted using a 4 × 250 mm Ion Pac® AS16 anion-exchange column packed with anion exchange resin. A Dionex ASRS® (Anion Self-Regenerating Suppressor) was used at 242 mA. The Dionex EG40 eluent generator equipped with a potassium hydroxide (KOH) cartridge was used in conjunction with the ASRS®. The newly developed EG40 eluent generator system electrolytically produces high-purity KOH eluent using deionised water as the carrier stream at the point of use. The use of the EG40 hydroxide eluent generator resulted in negligible baseline shifts during the hydroxide gradients, along with greater retention time reproducibility. The sample run time was set for 20 min where the gradient program initiated from 30 mM KOH for 10 min, then increased from 30 to 60 mM KOH over 5 min, followed by remaining at 60 mM KOH for 3 min, and finally the KOH returned to 30 mM for 2 min. Standard solutions were prepared using sodium phosphate tribasic, trisodium trimetaphosphate, pentasodium tripolyphosphate (Sigma-Aldrich, UK) and tetrasodium pyrophosphate (BDH, UK). A 100 ppm working solution containing all of the above four reagents was prepared, and serially diluted to 50, 20, 10 and 1 ppm standard solutions.

pH change

The pH of the post-degradation medium was measured using an Orion pH meter (Orion, UK) with a pH glass electrode (BDH, UK). The meter was calibrated using colourkey standard solutions (BDH, UK) every time before use.

Biological assessment

In vitro attachment and viability study of human osteosarcoma cell line (MG63) were conducted up to 7 days to assess the biocompatibility of TiO_2 containing glasses and compared with both TiO_2 free glass and Thermanox® positive controls. All samples for biocompatibility study were sterilised by heating at 180 °C for 3 h, and pre-treated by

incubation in a growth medium described below for 24 h at 37 °C humified atmosphere incubator of 5% CO_2 in air.

Cell culture. MG63 cells were cultured at 37 °C humified atmosphere incubator of 5% CO_2 in air, in a growth medium [Dulbecco's modified Eagles Medium (DMEM, Gibco), 10% fetal calf serum, and 1% penicillin and streptomycin solution (Gibco)]. The medium was changed every three days. For attachment and viability study, the cells were seeded on the surface of the glass discs of various compositions and Thermanox® at a density of 25×10^3 cells/disc in a 6-well culture plates.

Scanning electron microscopy. Samples for SEM were overnight fixed with 3% glutraldehyde in 0.1 M sodium cacodylate buffer (Agar Scientific Ltd., Essex, UK) at 4 °C, then dehydrated in graded alcohol (20, 50, 70, 90 and 100%). The dehydrated samples were critically dried in hexamethyldisilazane (HMDS, Taab Laboratories Ltd., Berkshire, UK) for 5 min, and then left to air dry. The dried samples were then mounted on aluminium stub, sputter coated with gold-palladium alloy, and viewed using a Stereoscan 90B Scanning electron microscope (Cambridge instruments Ltd., UK).

Cell viability and live dead staining. Determination of cell viability was carried out by incubating the discs and the controls for 1 h in a standard growth medium containing 1 μL/mL calcein AM, to stain the live cells, and propidium iodide, to stain the dead cells. Live cells cleave membrane-permeant calcein AM to yield cytoplasmic green fluorescence; membrane-impermeant propidium iodide labels nucleic acids of membrane compromised cells with red fluorescence. The assessment of cell viability in three dimensions was performed using confocal microscopy (Bio-Rad, USA). In a typical scan, the sample was placed into the bottom of a 35 mm culture dish and sections of the sample were scanned using a 20× lens. The region of interest was 600 μm × 600 μm, *x*–*y* dimension, and the images were collected at 2 μm intervals through the thickness of the cell sheet formed on the top of the surface in z-dimension (z-stacks) using Laser Sharp 2000 software. Excitation wavelengths for the fluorescent dyes for live and dead cells were provided at 488 nm from an argon laser and 543 nm from a Green/HeNe laser, respectively. Projection images were created by superimposing the z-stack images that were captured throughout the construct thickness using ImageJ software (National Institute of Health).

Statistical analysis

Student's *t*-test was used to study the effect of TiO_2 content on density and the glass transition temperature. Significance was detected at a 0.05 level, and all statistical analysis was carried out using the SPSS system for Windows (SPSS 12.0.1).

Results and discussion

This study investigated doping highly degradable ternary phosphate glasses having composition (in mol%) 50 P_2O_5, 30 CaO, 20 Na_2O with TiO_2 in order to produce glasses with controlled degradation and enhanced biological response. These glasses developed for potential application in bone tissue engineering applications. For this purpose, TiO_2 was added at the expense of Na_2O at 0, 1, 3 and 5 mol%. The density, thermal, structural, degradation, ion release and the resultant pH changes in an aqueous environment were used as tools to study the effect of TiO_2 incorporation on the glass structural and physical properties. The biological response of these glasses was also evaluated by means of MG63 human osteosarcoma cell biocompatibility.

Density

Figure 1 shows the density in g cm^{-3} of phosphate glasses as a function of TiO_2 content. The density of bulk glass was observed to be significantly ($p < 0.05$) increased with increasing TiO_2 content. It increased from 2.58 ± 0.002 to 2.63 ± 0.001 g cm^{-3} by incorporation of 5 mol% TiO_2 into the ternary glass formulation (0 mol% TiO_2). This may be attributed to the formation of a denser glass structure associated with close packing of atoms by the strong P–O–Ti bonds [16]. The density is known to be an important tool to measure the cross-link density and the compactness or close packing of atoms in the glass structure. Therefore, it gives an indication of the degree of the change in the glass structure with the change in the glass composition associated with the addition of modifying metal oxides.

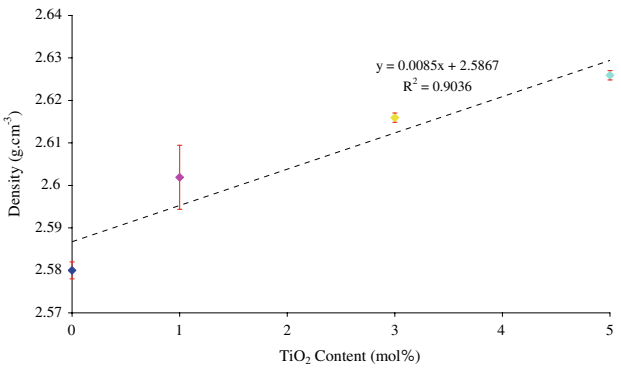

Fig. 1 Density (g cm^{-3}) as a function of TiO_2 content presented in mol%

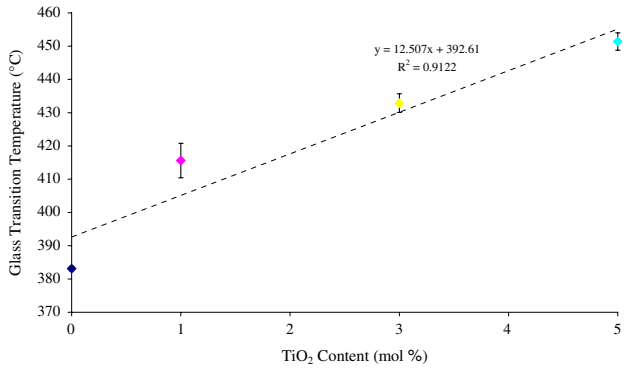

Fig. 2 Glass transition temperature (°C) as a function of TiO_2 content as measured by differential scanning calorimetry

Thermal analysis

The increase in density was related to the increase in T_g obtained from DSC as shown in Fig. 2. The T_g was significantly ($p < 0.05$) increased from 383 ± 1 to 451 ± 3 °C with the addition of 5 mol% TiO_2 into the ternary glass composition. The observed increase in both density and T_g was believed to be due to the formation of TiO_5 or TiO_4 structural units and the formation of P–O–Ti bonds that forms ionic cross-linking between the non-bridging oxygen of two different chains that strengthen the glass structure [16–18].

Figure 3 shows the DTA trace of TiO_2 containing compared to ternary glasses. The thermal properties of glasses are important, as it can provide an overview on the possible transformations that the glass undergoes at different temperatures and also give valuable information on the morphological structure. The first shift in the base line observed in the DTA trace was attributed to the glass transition temperature, and the upward peaks are exothermic with the downward peaks being endothermic. The exothermic peaks are attributed to the crystallisation temperatures, and the endothermic peaks, however, define the melting temperatures. The DTA trace of the 0 mol% TiO_2 was characterised by the presence of a single sharp crystalline peak at 538 °C and two melting peaks at 720 and 755 °C. A second crystalline peak was observed by addition of 1, 3, and 5 mol% TiO_2 into the ternary glasses, however, disappearance of the second melting peak was observed for 3 and 5 mol% TiO_2. The two crystalline phases obtained for 1 mol% TiO_2 containing glass were identified at 561 and 660 °C, and the two melting peaks were identified at 738 and 770 °C. Incorporation of 3 mol% TiO_2 produced a shift in the crystallisation and melting to higher temperatures at 590 and 690 °C, but only one melting peak which appeared at 778 °C. A shift in the crystallisation peaks to higher temperatures was also observed for 5 mol% TiO_2 containing glass (627 and 727 °C) with the melting peak was identified at 776 °C. Therefore, the addition of TiO_2 into the ternary glasses not only produced a change in the number of crystalline and melting peaks but also produced a linear increase in crystallisation, and melting temperatures.

X-ray powder diffraction

For the ternary glass with no TiO_2, a $NaCa(PO_3)_3$ phase (ICCD no. 23-669) was identified from the Crystallographica database as previously shown in [9, 10]. For glasses with 1 and 3 mol% TiO_2, the same phase $NaCa(PO_3)_3$ was also identified. For glasses with 5 mol% TiO_2, two phases were identified which were $NaCa(PO_3)_3$ (ICCD no. 23-669) and titanium phosphate TiP_2O_7 (ICCD no. 36-1468).

Degradation study

Figure 4 shows the cumulative degradation presented as % weight loss/per unit area as a function of time for various TiO_2 containing and free glasses. This degradation study was carried in deionised water by changing the medium to avoid the precipitation that accompanied the static environment shown in a previous study [11]. The degradation

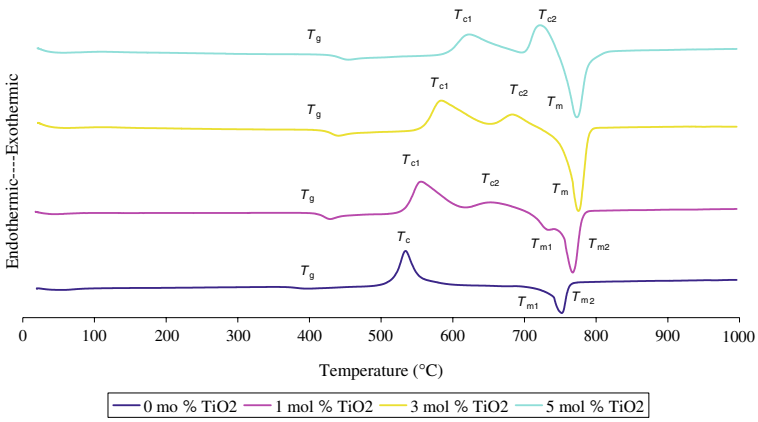

Fig. 3 DTA trace of TiO_2 containing glasses compared to TiO_2 free ternary glass

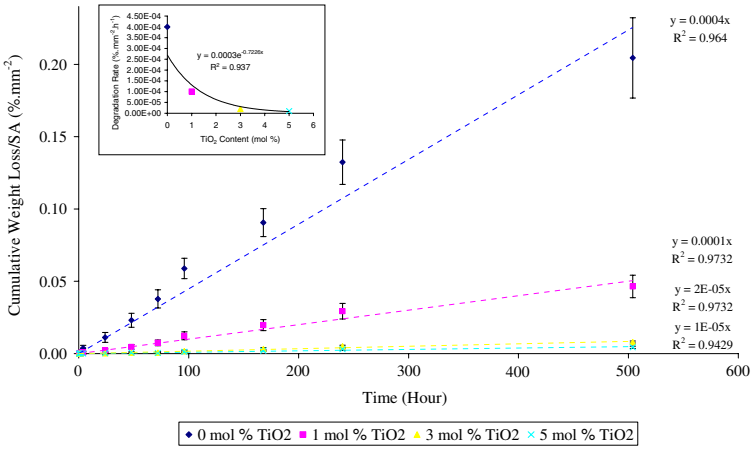

Fig. 4 Degradation presented as cumulative weight loss %/surface area for different TiO_2 containing glasses compared to TiO_2 free glass as a function of time. The inset represents the degradation rate (% mm^{-2} h^{-1}), calculated from the slope of the linear fit of cumulative weight loss %/surface area against time, as a function of TiO_2 content

process increased over time for all tested glass compositions, and it was decreased with increasing the TiO_2 content. The degradation rate was calculated from the slope of the linear fit of cumulative degradation against time. By plotting the degradation rates of the tested compositions against the TiO_2 content, an exponential relationship was produced as shown in the inset in Fig. 4. The range of the degradation rate presented as % mm^{-2} h^{-1} obtained in this study was 4×10^{-4} to 1×10^{-5}% mm^{-2} h^{-1} which indicates that the addition of TiO_2 up to 5 mol% produced a decrease in degradation rate by one order of magnitude. The observed reduction in the magnitude and rate of degradation with increasing TiO_2 content could be due to the formation of a highly cross-linked dense structure that resist degradation as evidenced from the T_g and density data.

Ion chromatography

Figure 5(a, b) shows the cumulative cation release in terms of sodium (Na^+) and calcium (Ca^{2+}) as a function of time for the studied glass compositions. As can be observed there was an increase in the release of both ions over the time of the experiment; this behaviour was observed for all tested glass compositions. This release trend mirrors the degradation behaviour, and the rate of ion release was also calculated from the slopes of the linear fit of the ion release against time. The rate of both Na^+ and Ca^{2+} ion release decreased with increasing TiO_2 content. The reduction in Na^+ release was expected due to the partial substitution of Na_2O with TiO_2 and thus a reduction in the amount of Na^+ available in the glass. Although all glass compositions contained 30 mol% CaO, there was a decrease in Ca^{2+} release with an increase in TiO_2 content. This result confirmed that the amount of both Na^+ and Ca^{2+} ion released was related to the degradation rate.

The cumulative release of different anionic species represented as orthophosphate (PO_4^{3-}) (Fig. 6a), pyrophosphate $P_2O_7^{4-}$ (Fig. 6b), cyclic trimetaphosphate

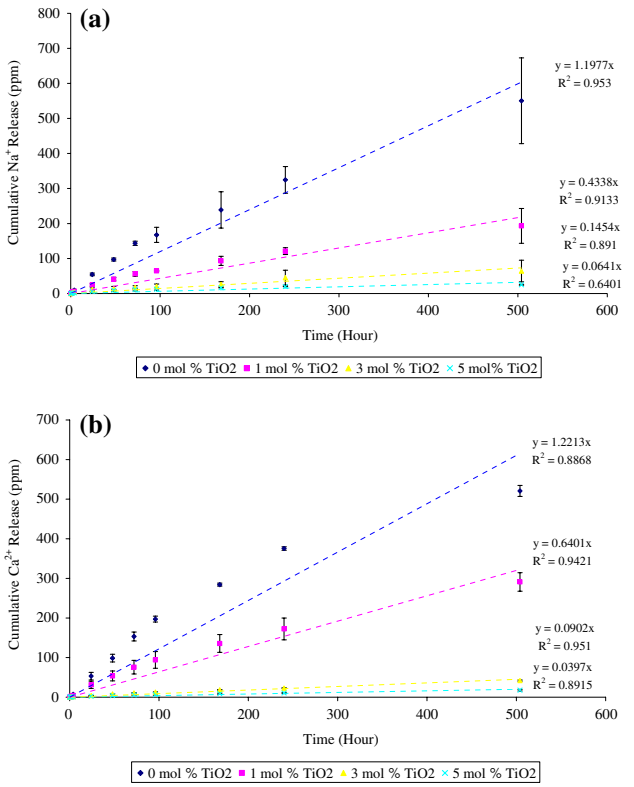

Fig. 5 Cumulative cation release (**a**) Na^+ and (**b**) Ca^{+2}, presented as ppm, as a function of time for different TiO_2 containing glasses compared to TiO_2 free glass

$P_3O_9^{3-}$ (Fig. 6c), and linear polyphosphate $P_3O_{10}^{5-}$ (Fig. 6d) was shown for all tested glass compositions. Similarly, the release also increased over time, and the release rate was considered from the slop of linear fit against time. All anionic release rates decreased with increasing TiO_2 content, and the highest release was observed for the $P_3O_9^{3-}$ anion when compared to the remaining identified species. This suggested that a significant proportion of the $P_3O_9^{3-}$ anion was present in the original glass structure as has been found for similar

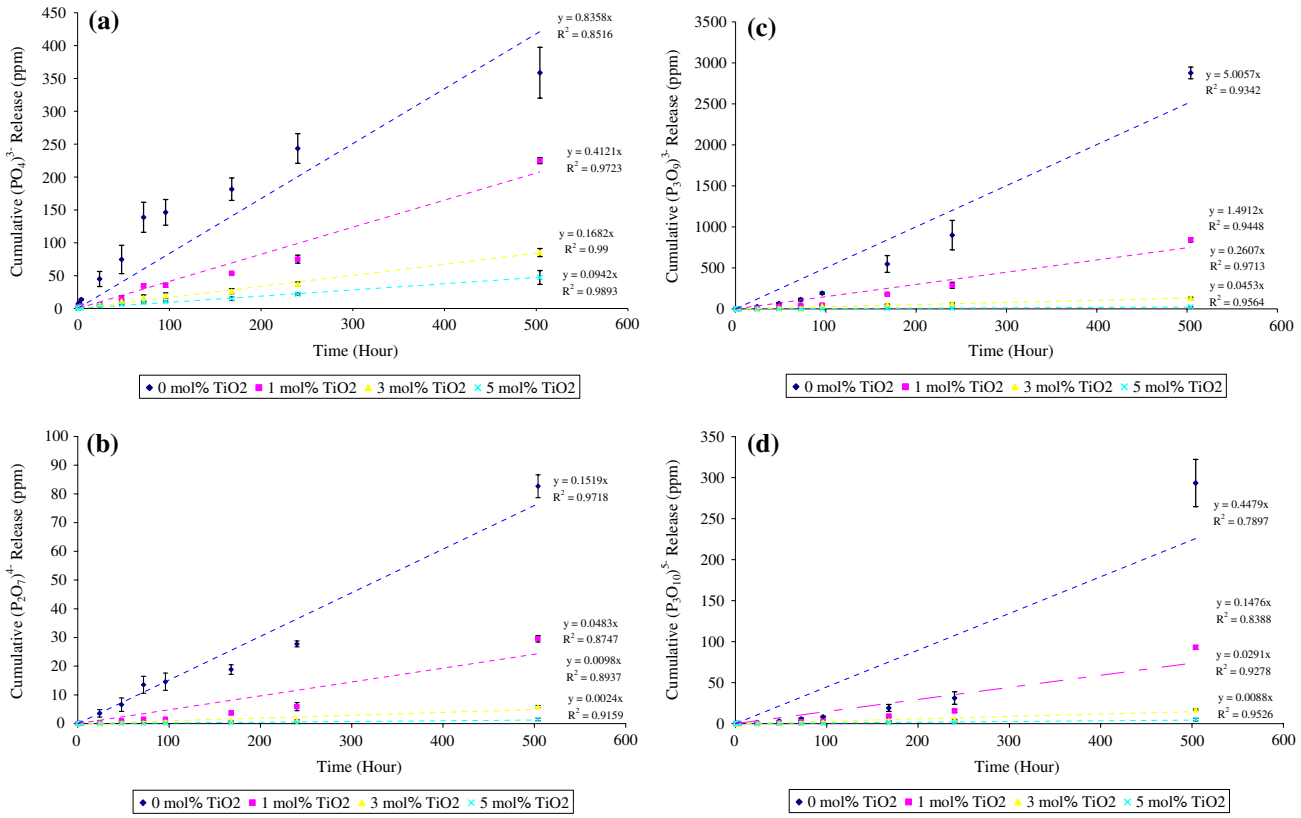

Fig. 6 Cumulative anion release (**a**) PO_4^{3-}, (**b**) $P_2O_7^{4-}$, (**c**) $P_3O_9^{3-}$ and (**d**) $P_3O_{10}^{5-}$, presented as ppm, as a function of time for different TiO_2 containing glasses compared to TiO_2 free glass

Table 2 Cumulative cation and anion release rate (ppm h^{-1}) calculated from the slope of the linear fit of ion release against time for the investigated glass compositions

Glass code	Cation (ppm h^{-1})		Anion (ppm h^{-1})			
	Na^+	Ca^{2+}	$(PO_4)^{3-}$	$(P_3O_9)^{3-}$	$(P_2O_7)^{4-}$	$(P_3O_{10})^{5-}$
0 mol% TiO_2	1.1977	1.2213	0.8358	5.0057	0.1519	0.4479
1 mol% TiO_2	0.4338	0.6401	0.4121	1.4912	0.0483	0.1476
3 mol% TiO_2	0.1454	0.0902	0.1682	0.2607	0.0098	0.0291
5 mol% TiO_2	0.0641	0.0397	0.0942	0.0453	0.0024	0.0088

ternary-based glasses [9, 11]. The release rate for cationic and anionic species presented as ppm h^{-1} is given in Table 2.

pH change

Figure 7 shows the associated change in pH of the deionised water in which the degradation study was carried out over the time of the experiment for all tested glass compositions. The pH values remained relatively neutral up to 10 days for glass discs with 3 and 5 mol% TiO_2, and then the pH dropped to around 6. However with 0 and 1 mol% TiO_2, the change in pH from a relatively neutral level was observed after 4 days, and the pH showed a rapid drop to

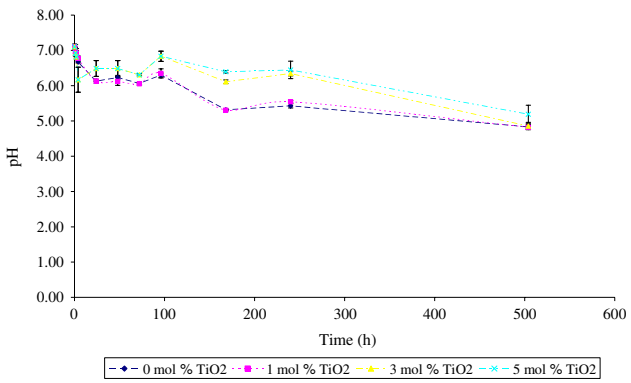

Fig. 7 pH change in deionised water as a function of time for different TiO_2 containing glasses compared to TiO_2 free glass

around 5. The drop in pH to acidic level may be due to the relatively higher degradation nature of these compositions compared to 3 and 5 mol% TiO_2 containing glasses. This in turn reflected with the higher level of release of different phosphate species into the degradation medium; the dissociation of these species resulted in the formation of phosphoric acid that produced an acidic environment [19].

Biological assessment

In this study, the osteoblast cell line MG63 was used to evaluate the biocompatibility of the material. These cells are derived from human osteosarcoma; they were selected because their extensive use in the investigation of osteoblast response to various biomaterials as they express a number of features characteristics of osteoblasts [20–22]. The biocompatibility of the glass was evaluated by examining MG63 cell attachment and viability in response to the glass surfaces.

Figure 8 shows scanning electron microscopy of MG63 cells attached to the surfaces of the tested glass compositions and a positive control after 1 day (a–e), 3 days (f–j), 4 days (k–o) and 7 days (p–t). At day 1, the MG63 cells had typically round morphology on TiO_2 free glass discs, but they were observed to have well spread three-dimensional morphology on TiO_2 containing glass discs of different compositions. Cellular processes can be clearly seen attaching to the surface of the discs with 1 mol% TiO_2, and the cells formed a flat monolayer over 3 and 5 mol% TiO_2 containing discs comparable to the Thermanox® positive control. It can also be inferred that fewer MG63 cells attached to the surface of 1 mol% TiO_2 discs compared to 3 and 5 mol%. This may explain the prominent appearance of cell processes on 1 mol% surface compared to 3, 5 mol% TiO_2 containing discs, and the Thermanox® positive control.

After three days, the MG63 cells still maintained the rounded morphology on TiO_2 free glass discs. However, fewer cells remained attached to 1 mol% TiO_2 glass discs compared to 1 day discs. The glass discs with 3 and 5 mol% TiO_2 were still comparable to the positive control by maintaining more attached cells with the characteristic spread morphology. This behaviour was maintained up to 7 days for MG63 attached on all tested compositions. These findings suggest that the TiO_2 containing glass discs, 3 and 5 mol% in particular, had profound effects on MG63 up to the time of the experiment compared to TiO_2 free composition. This is in agreement with the findings of Navarro et al. [23] who stated that incorporation of TiO_2 into 3D macroporous calcium phosphate glass ceramics had no cytotoxic effect on osteosarcoma cells using both direct and indirect contact test; and the cell viability was significantly higher than polystyrene culture plate used as a positive control. Generally, the observed relative reduction in cell attachment for 1 mol% TiO_2 compared to 3 and 5 mol% can be explained by the relatively higher degradation combined with the rapid reduction in the surrounding pH to acidic level. The relative similarity in MG63 behaviour on both 0 and 1 mol% TiO_2 could by due to the relatively high degradation rates of these composition compared to 3 and 5 mol% TiO_2 glasses. This was also confirmed by the similarity in the trend of pH changes for both compositions.

The cell viability was assessed using live/ dead fluorescent stain, and the samples viewed under confocal microscopy. This fluorescence-based staining was used to

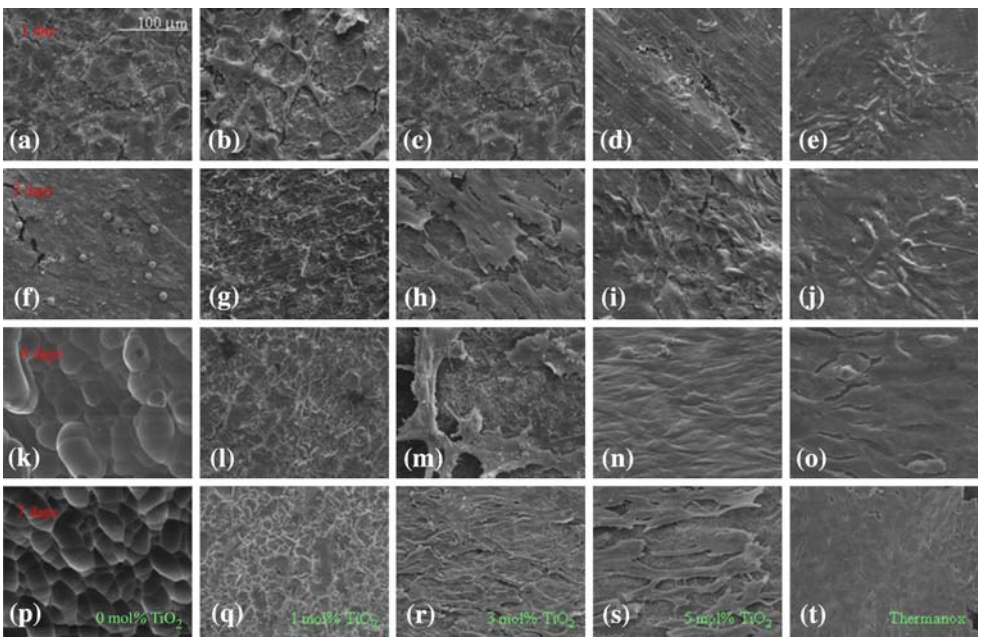

Fig. 8 Scanning electron microscopy showing the attachment of MG63 seeded on the surface of glass discs with different TiO_2 content compared to TiO_2 free glass discs and Thermanox® positive control after 1 day (**a–e**), 3 days (**f–j**), 4 days (**k–o**) and 7 days of culture (**p–t**)

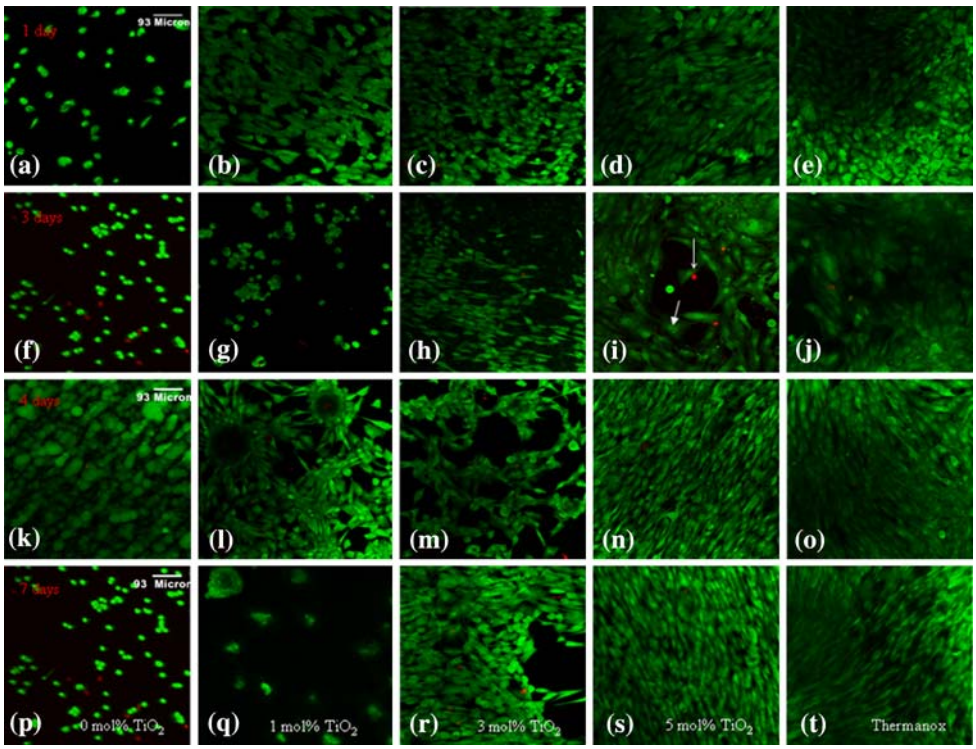

Fig. 9 Confocal images showing viability of MG63 seeded on the surface of glass discs with different TiO_2 content compared to TiO_2 free glass discs and Thermanox® positive control after 1 day (**a–e**), 3 days (**f–j**), 4 days (**k–o**) and 7 days of culture (**p–t**). The *arrows* refer to the presence of dead cells that fluoresce red and live cells that fluoresce green

differentiate between live and dead cells. Where live cells stained with calcein acetoxymethyl ester (AM) and fluoresce green, while dead cells stained with propidium iodide and fluoresce red [24]. Figure 9 shows confocal images of live/dead stained MG63 cells attached to the surfaces of the tested glass compositions and a positive control after 1 day (a–e), 3 days (f–j), 4 days (k–o) and 7 days (p–t).

After 1 day of culture, there were no dead cells detected on all glass surfaces, and this was comparable to the positive control. After 3 days, dead cells as well as the live cells were attached to all surfaces; yet 3 and 5 mol% TiO_2 containing discs maintained high cell viability observed by few dead cells coupled with high viable cell that totally cover the glass surface, and these compositions were still comparable to the positive control. This behaviour remained the same up to 7 days except for 1 mol% TiO_2 containing discs that showed an increase in cell viability at 4 days that dropped again by 7 days. The reduced viability on 1 mol% TiO_2 containing discs at 7 days was observed by the presence of fewer live cells attached to the surface, but as can be observed that no dead cells were observed at this time point which could be due to detachment of these dead cells into the medium.

The observed similarity in the biological response (cell attachment and cell viability) between 3 and 5 mol% TiO_2 could be due to the structural similarity in composition, since they possessed the same number of crystalline and melting peaks identified by DTA (Fig. 3). Alternatively, 1 mol% TiO_2 showed a significant structural difference in having a second melting peak that was not identified for 3 and 5 mol% TiO_2 containing compositions.

Conclusion

This study reported on the addition of TiO_2 as a dopant to control the degradation and biological behaviour of ternary phosphate glasses having the following formula; (in mol%) 50 P_2O_5, 30 CaO, 20 Na_2O. The degradation study was conducted in deionised water using a cumulative release method where the water was changed at regular intervals to avoid the precipitation processes sometimes associated with the static environment. The production of phosphate glasses with reduced degradation rates were successfully obtained by incorporation of TiO_2 up to 5 mol%. This may be due to the formation of TiO_5 or TiO_4 structural unit and the strong Ti–O–P bonds, which reflected in the glass thermal and structural properties where a linear increase in both density and T_g was observed. Moreover, a change in the number of crystalline phases was also observed by introduction of TiO_2 into the glass structure. The cation release profiles exhibited similar trends to that of degradation rates, as a decrease in both magnitude and rate of Na^+ and Ca^{2+} release was observed with increasing TiO_2 content. Similar trends were also obtained for the four identified anionic species (PO_4^{3-}, $P_2O_7^{4-}$, $P_3O_9^{3-}$, and $P_3O_{10}^{5-}$); the highest release rate was obtained for the $P_3O_9^{3-}$ species. This suggests that this anionic species

dominated the glass degradation process, and there was a significant proportion of this species in the original glass structure as previously observed [9, 11]. The pH of the post degradation medium was shown to be relatively neutral for both 3 and 5 mol% TiO_2 glasses; however, a rapid change in pH to acidic conditions was observed for both 0 and 1 mol% TiO_2 glasses. This was also reflected in cell attachment and viability where these two compositions showed relatively less favourable response compared to the remaining compositions. Conversely, both 3 and 5 mol% TiO_2 supported MG63 attachment and maintained high cell viability up to 7 days in a manner comparable to the Thermanox® positive control. It can be concluded therefore that phosphate glasses with 3 and 5 mol% TiO_2 could be a successful substrate for bone tissue engineering applications.

Acknowledgement The authors would like to acknowledge the EPSRC for providing the funding to conduct this study.

References

1. J. J. MARLER, J. UPTON, R. LANGER and J. P. VACANTI, *Adv. Drug Deliv. Rev.* **33**(1–2) (1998) 165
2. F. R. A. J. ROSE and R. O. C. OREFFO, *Biochem. Biophys. Res. Commun.* **292**(1) (2002) 1
3. K.-J. WALGENBACH, M. VOIGT, A. W. RIABIKHIN, C. ANDREE, D. J. SCHAEFER and G. B. STARK, *Anatom. Rec.* **263**(4) (2001) 372
4. D. C. CLUPPER, J. E. GOUGH, P. M. EMBANGA, I. NOTINGHER and L. L. HENCH, *J. Mater. Sci. Mater. Med.* **15** (2004) 803
5. J. E. GOUGH, I. NOTTINGHER and L. L. HENCH, *J. Biomed. Mater. Res.* **66A** (2004) 640
6. R. M. DAY and A. R. BOCCACCINI, *J. Biomed. Mater. Res.* **73A**(1) (2005) 73
7. M. CERUUTI, D. GREENSPAN and K. POWERS, *Biomaterials* **26** (2005) 1665
8. J. C. KNOWLES, *J. Mater. Chem.* **32** (2003) 395
9. I. AHMED, C. A. COLLINS, M. LEWIS, I. OLSEN and J. C. KNOWLES, *Biomaterials* **25** (2004) 3223
10. E. A. ABOU NEEL, I. AHMED, J. PRATTEN, S. N. NAZHAT and J. C. KNOWLES, *Biomaterials* **26** (2005) 2247
11. E. A. ABOU NEEl, I. AHMED, J. J. BLAKER, A. BISMARCK, A. R. BOCCACCINI, M. P. LEWIS, S. N. NAZHAT and J. C. KNOWLES, *Acta Biomater.* **1** (2005) 553
12. J. E. GOUGH, P. CHRISTIAN, C. A. SCOTCHFORD and I. A. JONES, *J. Biomed. Mater. Res.* **66A** (2003) 233
13. M. BITAR, V. SALIH, V. MUDERA, J. C. KNOWLES and M. LEWIS, *Biomaterials* **25** (2004) 2283
14. V. SALIH, K. FRANKS, M. JAMES, G. W. HASTINGS and J. C. KNOWLES, *J. Mater. Sci. Mater. Med.* **11** (2000) 615
15. N. MORTIZ, E. VEDEL, H. YLANEN, M. JOKINEN and M. HUPA, *J. Mater. Sci. Mater. Med.* **15** (2004) 787
16. P. K. BROW, D. R. TALLANT, W. L. WARREN, A. MCINTYRE and D. E. DAY, *Phys. Chem. Glasses* **38**(6) (1997) 300
17. V. RAJENDRAN, A. V. GAYATHRI DEVI, M. AZOOZ and F. H. EL-BATAL, *J, Non-Cryst. Solids* **353**(1) (2006) 77
18. T. KASUGA and Y. ABE, *J. Non-Cryst. Solids* **243** (1999) 70
19. J. R. V. WAZER and K. A. HOLST, *J. Am. Ceram. Soc.* **72** (1950) 639
20. J. J. BLAKER, J. E. GOUGH, V. MAQUET, I. NOTINGHER and A. R. BOCCACCINI, *J. Biomed. Mater. Res.* **67A** (2003) 1401
21. L.-L. TSENG, C.-J. HUANG, S.-S. HSU, J.-S. CHEN, H.-H. CHENG, H.-T. CHANGE, B.-P. JIANN and C.-R. JAN, *Clin. Exp. Pharmacol. Physiol.* **31** (2004) 732
22. S. J. LEE, J. S. CHOI, K. S. PARK, G. KHANG, Y. M. LEE and H. B. LEE, *Biomaterials* **25**, (2004) 4699
23. M. NAVARRO, S. D. VALLE, S. MARTINEZ, S. ZEPPETELLI, L. AMBROSIO, J. A. PLANELL and M. P. GINEBRA, *Biomaterials* **25**, (2004) 4233
24. M. NAVARRO, S. D. VALLE, S. MARTINEZ, S. ZEPPETELLI, L. AMBROSIO, J. A. PLANELL and M. P. GINEBRA, *Biomaterials* **25**, (2004) 4233