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3. Article title: **Physical Properties and Biocompatibility Effects of Doping SiO_2 and TiO_2 into Phosphate-Based Glass for Bone Tissue Engineering**

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5. Abstract

Abstract

Phosphate glass is gaining more attention to serve as a bone substitute. More specifically, the ternary (P_2O_5 -CaO-Na_2O) was investigated in terms of both physical properties and biocompatibility by doping different percentages of SiO_2 and TiO_2. Disks from two groups of different compositions of the ternary (P_2O_5-CaO-Na_2O) were prepared. The first group has different percentages of TiO_2 and SiO_2, whereas the second group compositions has 5mol% TiO_2 and 5mol% SiO_2 added to compensate the network-forming oxide P_2O_5 and the network-modifying oxide CaO.

Density, degradation, DTA, XRD, and cation release experiments were performed to study the physicochemical properties of the compositions, while MG63 and hMS cells were used within *in vitro* cell culture studies to study their biocompatibility. Results showed that a decrease in SiO_2 content directly correlated with an increase in glass density, decreased degradation, increased trend of Tg and Tm values, and Na^+ and Ca^{2+} release in group 1. However, there was no improvement in the MG63 viability or the ability of hMSC's to differentiate into osteoblasts where TiO_2 was replaced with SiO_2. Furthermore, in group 2, 50P_2O_5-25CaO was less dense than 45P_2O_5-30CaO, degraded dramatically less, had lower Tg and Tm values and released less $Na^+$and Ca^{2+}. The synergistic effect of doping 5mol% TiO_2 and 5mol% SiO_2 increased the MG63 viability in both compositions and was found 45P_2O_5-30CaO to have superior results in terms of the ability of hMSC's to differentiate into osteoblasts. To conclude, substituting TiO_2 in place of SiO_2 improved the physical properties and the

biocompatibility of (P_2O_5-CaO-Na_2O) glass system whereas doping 5mol% SiO_2 and 5mol% TiO_2 together in place of P_2O_5 and CaO had a synergistic effect in controlling their degradation rate and improving their biological responses.

Key words: Tissue Engineering, Bone Augmentation, Synthetic Bone, Phosphate-based Glass, Silicate-based Glasses.

1. Introduction.

Regenerative medicine is focused on developing materials that can help clinicians to manage hard tissue loss. This is vital to patients suffering from trauma, neoplasms and congenital abnormalities, which can be associated with significant scarring and deformation with potential psychological impairments. The concept of hard tissue augmentation went through different phases of techniques and materials until formulating the concept used today.

Autogenous graft is still considered to be the gold standard of bone grafting materials (1). However, different synthetic materials have been studied and used extensively in research and later in clinical environments such as silicate-based glasses, phosphate and borosilicate based glasses and hydroxyapatite. Silicate based glasses or the now famous 45S5® Bioglass were introduced by Larry Hench in the late 1960s as a revolution in the development of biomaterials and bone tissue engineering (1). Its bioactive properties made it a potential material to be applied clinically for bone defect repair (2). Its favourable properties granted it great attention in tissue engineering research as it is biodegradable, has the ability to drive the differentiation of hMSC's and their proliferation into osteoblasts (1, 3-5). Phosphate glasses, in turn, are also considered good candidate materials for both bone tissue engineering and also can be antimicrobial (6, 7). This can be attributed to two main factors; the controllability of its degradation and the similarity of its inorganic composition, such as P, Na and Ca, to the human bone (8, 9). Although it has a hygroscopic nature, it has been modified by doping with many metal oxides such as TiO_2, GeO_2 and B_2O_3 (6, 10). An example of such glass is the ternary (P_2O_5– CaO – Na_2O) which was developed and modified to control its degradation rate. Adding TiO_2 might have contributed to

increasing the covalent nature of the bonds within the glass structure as well as playing a major role in attracting osteoblasts and improving their attachment and proliferation (6). This attracted more attention to this ternary system as a potential material for bone engineering application.

The present paper focuses on doping SiO_2 and TiO_2 into phosphate-based glass (P_2O_5-CaO-Na_2O). The main goal is to control their degradation rate and enhance their biological response to be suitable synthetic materials that can be applied in bone augmentation. The concept behind the choice of SiO_2 to serve as an additive oxide to phosphate-based glass (P_2O_5-CaO-Na_2O-TiO_2) was due to the biocompatibility of silicate-based glasses as shown in many previous studies, and the good results obtained from doping Mg and Al in similar researches (11, 12).

As Si shares a position on the third row of the periodic table with Mg, Al and Na, it was suggested to investigate doping it into quaternary based-glass. Three different aspects are discussed in this paper, which include the production of different phosphate glass compositions, their physicochemical properties, and their biocompatibility reaction within an in *vitro* environment using human osteoblast-like osteosarcoma cells (MG63) and human mesenchymal stem cells (hMSC's).

2. Materials and Methods.

2.1.Materials Preparation

Eight different glass rods of 15mm diameter were prepared using eight different compositions of sodium dihydrogen phosphate NaH_2PO_4, calcium carbonate $CaCO_3$, phosphorus pentoxide P_2O_5, titanium dioxide TiO_2, and silicon dioxide SiO_2 (BDH, Poole, UK, all chemicals were >98% purity) as shown in table 1.

The first group has six compositions with similar core components (50% P_2O_5-30% CaO -15% Na_2O) mol% though different TiO_2 to SiO_2 percentage, starting from (5% TiO_2 / 0% SiO_2) and gradually replacing TiO_2 with SiO_2 to reach (0% TiO_2 / 5% SiO_2). The second group has two compositions both contain (5% TiO_2 / 5% SiO_2) with different percentages; (45% P_2O_5-30% CaO-15% Na_2O-5% TiO_2-5% SiO_2) and (50% P_2O_5-25% CaO-15% Na_2O-5% TiO_2-5% SiO_2). The precursors were mixed thoroughly (Stomacher 400 Circulator, Seward, Worthing, UK) and then placed in a 200 ml crucible Pt/10%Rh type 71040 (Johnson Matthey, Royston, UK). The conventional melt quenching technique was used to prepare the glass rods.

Each composition was prepared and mixed, and then heated in two stages. The first cycle was at 750℃ for 30 minutes aiming to eliminate CO_2 and H_2O, whereas the second cycle was at 1350℃ for four hours to ensure chemical homogeneity. The melt then was poured into a 15mm diameter mold and left to cool down gradually at 250℃ for 30 minutes and the furnace was then switched off and the mould and glass allowed to cool down over the next 24 hours in order to remove residual stress. Then, each rod was sectioned into approximately 2mm thick discs using a Testbourne diamond saw with ethanol as a coolant. Discs from each composition were subjected to a series of grinding steps on two sides using a series of waterproof silicon carbide papers: P no. 120 for 30 seconds at 300RPM to flatten the surface, then P no. 500, 1000, and 2400, respectively, for 1min at 500RPM to smoothen the surfaces, and finally P no. 4000 for 2 min at 500RPM to get a smooth mirror-finish surface (Struers, UK).

Table 1 the amounts of precursors used in the eight different compositions.

2.2. X-ray Powder Diffraction (XRD)

This rapid analytical technique was applied to ensure the glasses were fully amorphous and was measured by X-ray diffractometer (D8 Advance Diffractometer, Bruker, Coventry, UK). The specimens were finely ground for 10 minutes and then positioned in flat plate geometry and (Ni-filtered Cu Kα) radiation was applied. Data were collected using a Lynx Eye detector with a step size of 0.02 over an angular range of 10–100° 2ϑ and a count time of 12 seconds.

2.3. Density

An analytical balance and density determination apparatus with respect to Archimedes' principle were applied (Mettler Toledo, UK). Triplicate samples of each composition were used to calculate the density using the equation below:

$$\rho\ glass = \frac{mass\ in\ air\ (g)}{mass\ in\ air - mass\ in\ ethanol}\ x\ \rho\ ethanol$$

where $\rho\ ethanol$ is the density of ethanol in g.cm^{-3} at the temperature of which the measurements of $m\ air$ in g and $m\ ethanol$ in g were performed. In our experiment the temperature was 20°C and ethanol used was 99.8% (Sigma Aldrich, Gillingham, UK) hence $\rho\ ethanol$ = 0.781596 g.cm^{-3}.

2.4. Differential Thermal Analysis

Setaram differential thermal analyser was used to perform differential thermal analysis (DTA) studies (Setaram, France). Approximately 60mg of glass powder (discs were grinded and the size of the particles varies between 30μm-150μm) were placed in a 100 μl platinum crucible prior to placing it in the analyser. The sample was then heated from room temperature to 1000℃ at a heating rate of 20℃ per minute using air as the purge gas. An empty platinum crucible was used as a reference. The parameters measured by DTA were glass transition temperature (Tg), crystallisation temperature (Tc) and melting temperature (Tm).

2.5. Degradation

Three discs from each composition were weighed and then stored in 5 ml deionized water (pH 7.0 $\pm$ 0.1) for 1, 4, 7 and 14 days. The ultrapure water was chosen parallel to the ion release experiment. This followed the protocol of previous studies. At each time point, the glass discs were taken out of the water, dried manually, weighed and then transferred into a fresh 5 ml of distilled water. In between the time points, samples were stored in a 37°C incubator. Mass change of each glass disc was calculated using the equation below:

$$\Delta m = \frac{m\,0 - m\,t}{A}$$

Where $m\,0$ is the initial mass (mg) of the disc on day 0, $m\,t$ is the mass (g) of the disc at specific time point and A is the surface area (cm^2) of the glass disc. In our experiment r(disc) = 0.75 cm, hence $A = 2\pi$ rh + 2 π r^2 which is equal to about 4.45 cm^2.

2.6. pH Changes

Three discs from each composition were stored in 1 ml Dulbecco Modified Eagles Medium (DMEM) (Gibco®, Life Technologies Ltd., Paisley, UK), pH 8.4± 0.1, for 1, 4 and 7 days. At each time point, the pH of the specimen extract was measured using pH detector; Orion Star pH Meter (Thermo Scientific, UK). The culture media was changed every 3 days to mimic the environment of the cell culture study. Samples were stored in a 37°C/5% CO2 incubator in between time points. The culture media without discs was used as control.

2.7. Ion Chromatography

The solutions collected above for the degradation measurements were collected for ion determination. The ultrapure water was chosen to eliminate the presence of any background readings might be created if any buffered solutions (e.g simulated body fluid) was used. This helped to focus on the ions of interest only. Due to the high levels of ions such as Ca^{2+}, Na^{+} and P^{5+} in buffer solutions precluding the determination of the relavtively low levels of ion release form the glass, the measurements were performed in ultrapure water. For cation determination, as the separator column possesses high sensitivity to phosphate species, all samples were filtered with OnGaurd IIA filters (Dionex, Thermo Scientific, Hemel Hempstead, UK). Prior to performing the experiment, the system was calibrated against a five-point calibration curve using a previously developed routine before carrying out the sample run for each time point. $Na^{+}$and Ca^{2+} ion release measurements were performed on a Dionex ICS-1000 ion chromatography system (Dionex, Surrey, UK) using 30 mM methanesulphonic acid (Fluka, Dorset, UK) incorporating a 4 x 250 mm Ion Pac® CS12A separator column and a sample loop of 25 µl.

Sodium chloride (Sigma–Aldrich, Dorset, UK) and calcium chloride (BDH, Poole, UK) were used as reagents to prepare a 100-ppm stock solution before serially diluting both to produce 50, 25, 10 and 5-ppm standard solutions

2.8. In *Vitro* Cytocompatibility Assessment

Samples for the biocompatibility test were cleaned with ethanol 98% and then sterilised on both sides using ultraviolet light for 10 minutes.

2.8.1. Human Osteoblast-Like Osteosarcoma Cells (MG-63)

Human osteoblast-like osteosarcoma cells MG63 were obtained from in-house stocks at the Eastman Dental Institute, University College London. Two different assays were applied; Alamar Blue and Live/Dead assay with confocal laser scanning microscopy. Cells were prepared and then seeded calculating a density of 3000 cells per cm^2 in 1 ml of medium. Then, three discs from each composition were placed in a conventional 24 well tissue culture test plate (Transwell® polyester inserts, Corning, USA) with one disc per well. Seeding of cells on the discs was carried out calculating 5300 cells in each well.

2.8.2. Human Mesenchymal Stem Cells (hMSc)

Human mesenchymal stem cells were obtained from in-house stocks at the Eastman Dental Institute, University College London. Two different assays were used which are alkaline phosphatase and cell counting Kit 8 (CCK). Cells were prepared and then seeded calculating a density of 3000 cells per cm^2 of 1 ml medium. Then, three discs from each composition were placed in a conventional 24 well tissue culture test plate (Transwell®

polyester inserts, Corning, USA) as in one disc per well. Seeding of cells on the discs was carried out calculating about 5000 cells in each well.

2.7.3 Statistical Analyses

All statistical analyses were performed using Microsoft Excel 2013 and IBM SPSS version 22 (SPSS Inc., USA). Initial normality tests showed that the data of the cell culture studies were not normally distributed; hence they were restructured using the square root to apply normal distribution tests. Consequently, Bonferroni correction method and Bunnet test were applied. The assumed level of significance for the statistical tests was 0.05.

3. Results

3.1 X-ray powder Diffraction (XRD)

XRD patterns of the glass were measured. The broad peak confirmed the amorphous nature of all glass samples in both groups and indicated the absence of crystalline phases as shown in figure 1.

Figure 1 the pattern of the samples.

3.2 Density

The results presented in table 2 ranged from 2.54g/cm^3 to 2.59g/cm^3 in both groups. It was calculated that an increase in TiO_2 content correlated with an increase in glass density in group 1. In parallel, $45P_2O_5$-30CaO was relatively denser than $50P_2O_5$-25CaO in group 2 with densities (ρ = 2.58 $\mp$ 0.016 and ρ = 2.55$\mp$ 0.003 g/cm^3) respectively.

3.3 Differential Thermal Analysis (DTA)

DTA studies were performed on glass powder. Table 2 shows that the Tg of the glasses was in the range of 421–481°C while Tm was between 778-878°C with a decreased trend observed as SiO_2 the content was increased in both groups. Tc, in turn, was in the range of 681-777°C with no specific trend noted.

Table 2 density (g/cm^3) and different temperature values Tg, Tc and Tm of all samples.

3.4 Degradation (Mass changes)

Mass changes of each glass disc were calculated at four time-points as shown in figure 2. After 14 days, in group 1 Ti0Si5 had the highest mass loss (m= 6.86 $\pm$ 0.99) mg.cm^{-2} followed by Ti1Si4, Ti2Si3, Ti3Si2, Ti4Si1 and Ti5Si0 respectively, whereas in group 2, 45P_2O_5-30CaO had higher mass loss (m= 6.35$\pm$ 0.41 mg.cm^{-2}) than 50P_2O_5-25CaO. A comparison between the two groups showed that Ti0Si5 had the highest mass loss while Ti5Si0, Ti4Si1 and 50P_2O_5-25CaO were the least to be degraded with similar results (m= 0.77 $\pm$ 0.06, m= 0.98 $\pm$ 0.13, m= 1.39 $\pm$ 0.26) mg/cm^2. This indeed might have an impact on ion release and in *vitro* culture studies.

Figure 2 the trend of mean averages with standard deviations of mass loss on day 1, 4, 7 and 14.

3.5 pH Changes

As the culture media was changed at every time point within *in vitro* atmosphere, a similar approach was applied to calculate the change in pH. The culture media was changed after each time point to mimic the environment of the cell culture studies.

All pH values recorded were alkaline as presented in table 3. While the highest pH recorded for control was on day 4 (m=9.38 $\mp$ 0.24), the highest pH recorded for a composition was for 45 P_2O_5-30CaO on day 4 (m= 9.74 $\mp$ 0.17). The lowest pH recorded for control was on day 1

(m=8.38 ∓ 0.08), whereas the lowest pH recorded for a composition was for Ti0Si5 on day 1 (m=8.37 ∓ 0.22).

Table 3 the mean averages of pH on day 1, 4 and 7.

3.6 Ion Chromatography

All compositions released Na^+ and Ca^{2+} over the period of 7 days in a similar trend as shown in figure 3 and 4. In group 1, Ti0Si5 and Ti2Si3 released more $Na^+$and Ca^{2+} than the rest, whereas 45P_2O_5-30CaO released more $Na^+$and Ca^{2+} than 50P_2O_5-25CaO composition.

Figure 3 the mean average and standard deviations of Na^+cations release on day 1, 4, 7.

Figure 4 the mean average and standard deviations of Ca^{2+}cations release on day 1, 4, 7.

3.7 In *Vitro* Cytocompatibility Assessment

3.7.1 Alamar Blue Assay

Results of alamar blue showed that for the same composition, cells in all samples underwent proliferation up to 7 days (Figure 5). While on day 7 the control group had (n= 37127 ± 3672) cells, it was noticed that Ti5Si0 had the highest number of cells in group 1 m≈ 33043.10 cells. Other compositions were in between with Ti3Si2 and Ti4Si1 having higher cell numbers than Ti2Si3 and Ti1Si4. Whereas in group 2, both 50P_2O_5-25CaO and 45P_2O_5-30CaO had higher number of cells compared to the samples in group 1 (m= 34855, and 33547cells respectively) and

were similar to Ti5Si0.

Figure 5 the mean average and standard deviations of alamar blue assay on day 1, 4, 7.
* The mean difference is significant at the 0.05 level, 95% Confidence Interval.

3.7.2 Live/Dead Assay

Confocal Laser Scanning Microscopy (CLSM) images of MG63 cells attached to all samples were performed using live/dead stain on day 1, 4 and 7 post-culture. This is subjective and the main reason of choosing such an assay was to determine the viability of MG63 cells based on plasma membrane integrity and esterase activity (green for live cells/ red for dead cells). All compositions showed relatively good results at all three time-points as shown in figure 6.

Figure 6 CLSM images of MG63 cells attached to different composition 1,4 &7 days post culture under 20X-wet lens.

3.7.3 Alkaline Phosphatase Assay

Results of alkaline phosphatase presented in figure 7 showed that the control group recorded m= 0.343± 0.107 after 7 days. In group 1, Ti0Si5 and Ti1Si4 had significantly lower ALP values than the rest of the compositions (m= 0.163± 0.038, 0.220±0.053) respectively; while in group 2 both $50P_2O_5$-25CaO and $45P_2O_5$-30CaO had similar ALP values to the control group. On day 14, the situation had changed as Ti0Si5 and Ti1Si4 had similar ALP values to the control group however Ti5Si0, Ti4Si1, Ti3Si2 and Ti2Si3 had superior ALP values in comparison with both the control and the other compositions. In group 2, both $50P_2O_5$-25CaO and $45P_2O_5$-

30CaO had similar ALP values to the control group on day 7, however, on day 14 $50P_2O_5$-25CaO had significantly higher ALP values than both control and $45P_2O_5$-30CaO.

Figure 7 the mean average and standard deviations of Alkaline Phosphatase on day 7 & 14. *The mean difference is significant at the 0.05 level, 95% Confidence Interval.

3.7.4 CCK Assay

Results of the CCK presented in figure 8 showed that all samples had induced cell proliferation. While on day 1, Ti5Si0 and Ti3Si2 had a similar number of cells to the control group and higher compared to the rest of the compositions in group 1. This however had changed on day 4 and 7 as all samples had similar results and were lower than the control group. In group 2, both compositions had lower numbers of cells in comparison with the control group with $50P_2O_5$-25CaO having lower number of cells than $45P_2O_5$-30CaO though the difference was insignificant to the rest of the compositions.

Figure 8 the mean average and standard deviations of CCK assay over 1, 4 and 7 days. *The mean difference is significant at the 0.05 level, 95% Confidence Interval.

4. Discussion

4.1 Composition Selection

This paper focused on studying the effect of doping TiO_2 and SiO_2 into the ternary (P_2O_5 -CaO -Na_2O). While many studies (13-15) focused on adding different metallic oxides such as

TiO_2, Fe_2O_3 and SrO, into (P_2O_5 -CaO -Na_2O) glass system, this paper tried to combine doping two different oxides; metallic and nonmetallic. While compositions in group 1 were prepared to investigate the effect of adding different percentages of SiO_2 and TiO_2 to the system (50%P_2O_5-30%CaO -15%Na_2O), group 2 compositions were also studied to give a better understanding of this effect knowing the fact that silicate-based glass is more stable than the phosphate based version. Therefore, 5%SiO_2 and 5%TiO_2 were added into (50%P_2O_5-30%CaO-15%Na_2O) compensating the network-forming oxide in P_2O_5 (45%P_2O_5-30%CaO-15%Na_2O-5%TiO_2-5%SiO_2) and the network-modifying oxide in Ca (50%P_2O_5-25%CaO-15%Na_2O-5%TiO_2-5%SiO_2). The main aim was to create a more complex system, increasing the glass stability and improving the cytocompatibility.

4.2 X-ray Powder Diffraction (XRD)

X-ray diffraction was used to provide information about the structure of all compositions. Our results showed that all patterns had broad peaks, which confirmed the amorphous nature of all glass samples and indicated that they lacked a crystalline phase.

4.3 Density

In terms of group 1 compositions, their core components were the same (50%P_2O_5-30%CaO -15%Na_2O) with different SiO_2 to TiO_2 ratios. Results showed that an increase in TiO_2 content correlated with an increase in glass density. This was due to the fact that the relative atomic weight of titanium (47.867) is greater than silicon (28.085), hence Ti has a larger sample mass, whereas the atomic radius of silicon (117.6pm) is smaller than titanium (176pm); hence Si

has a reduced inter-atomic space. Both effects jointly led to an increase in glass density with the increase in TiO_2 content.

In terms of group 2 compositions, their core components were also the same (15%Na_2O-5%TiO_2-5%SiO_2) with different P_2O_5 to CaO ratios. Results showed that $45P_2O_5$-30CaO (ρ = 2.58 g.cm^{-3} $\pm$ 0.016) was slightly denser than $50P_2O_5$-25CaO (ρ = 2.55 g.cm^{-3} $\pm$ 0.003), which did not follow the trend of the relative atomic weight and the atomic radius described previously. For example, the relative atomic weight of calcium (40.078) is larger than phosphorus (30.973), whereas the atomic radius of phosphorus (98pm) is slightly smaller than calcium (118pm) and hence $50P_2O_5$-25CaO should be denser than $45P_2O_5$-30CaO; however, results were the opposite. The justification of such results may be attributed to the fact that the density may be controlled not only by the density of the element but also the bond lengths (16).

4.4 Differential Thermal Analysis (DTA)

DTA trends were obtained followed by identification of Tg, Tc and Tm values. Group 1 compositions had two changing variables; TiO_2 and SiO_2. Knowing the fact that the bond enthalpy of Ti−O (672 kcal/mol) is greater than Si−O (452 kcal/mol) may explain the decreased trend observed as the TiO_2 content was decreased and SiO_2 content was increased. On the other hand, group 2 compositions had also two changing variables; P_2O_5 and CaO. As Ca−O has a stronger ionic bond compared to the Van Der Waals bond between P=O….P=O, P_2O_5 needed higher energy to break these bonds.

Furthermore, the trend of Tm values was similar to those of Tg. This was due to the high melting point of titanium (1668°C) in comparison with other components. Consequently, the less TiO_2, the lower the melting point. Similarly, as $45P_2O_5$-30CaO contained more calcium than $50P_2O_5$-25CaO, its Tm was higher. This was also due to the stronger ionic bond in Ca−O in comparison with the Van Der Waals bond between P atoms. On the other hand, Tc readings did not follow any specific trend. This might be related to the differences in particle size (17).

4.5 Degradation

One of the main aims of doping SiO_2 and TiO_2 into phosphate glasses was to control their degradation. As glass degradation behaviour is closely linked to glass chemistry, alteration of the glass composition may allow a better control of the degradation rate over time (6). Results showed that degradation increased with decreasing titanium oxide and increasing silicon oxide in group 1 compositions. Therefore, Ti0Si5 degraded the most in comparison to other compositions. Ti1Si4, however, degraded slowly on day 1 before catching up with the rest of the compositions. These results concurred with previous studies (18).

Furthermore, results of group 2 compositions showed that $50P_2O_5$-25CaO degraded dramatically less than $45P_2O_5$-30CaO. Such results may be related to the fact that the latter has a lower percentage of network-forming oxide, but there is a possible more complex interaction at play. Although many studies indicated that solubility decreases with decreasing P_2O_5 and increasing CaO (19), this research has doped two different oxides in favour of the network-forming and the network modifying oxides. This might have alter the result suggesting new concept to be investigated in the future.

4.6 pH Changes

Although previous papers have used deionized water to detect pH changes, this paper used 1 ml of Dulbecco Modified Eagles Medium (DMEM), pH 8.4$\pm$ 0.1, as an immersion liquid. The main idea of this approach was to copy the conditions arranged for the cell culture study. After each time-point, the culture media for all glass discs was changed as well as triplicate of DMEM to act as a control. Samples were stored in a 37°C/5% CO_2 incubator in-between time-points. Our results concluded that the solutions obtained from all samples were found to be alkaline. In compare to control group, all samples had an increased pH values at all time points with the highest scores recorded on day 4 for all of them. The trend obtained from the sample values was similar to the one obtained from the control values.

4.6 Ion Chromatography

Results showed that all sample released Na^+ and Ca^{2+}cations over the period of 7 days. Complying with the results of degradation, Ti0Si5 and 45P_2O_5-30CaO released more Na^+ than the rest as they degraded the most, whereas Ti5Si0 and Ti4Si1 released the least as they were the less degraded samples. Similarly, Ti0Si5 and 45P_2O_5-30CaO released the most Ca^{2+}, while Ti5Si0 and Ti4Si1 released the least. Degradation and ion release proportionality was discussed thoroughly in the literature with all in agreement that the more degraded the sample is, the higher its ability to release ions.

4.7 In *Vitro* Biocompatibility Assessment

4.7.1 Alamar Blue Assay

This assay was used to investigate the interactions between cells and glass discs of all samples and to identify compositions that would elicit the most favourable cellular responses. Consequently, using different known numbers of cells with their alamar blue readings, an equation was derived to count the number of cells indirectly using the alamar blue readings obtained.

In compare to control, results of group 1 compositions showed that the alamar blue readings increased with decreasing SiO_2 and increasing TiO_2 after 7 days. Therefore, on day 7 Ti5Si0 had the highest values within this group (n=33043, P value = 0.205) while Ti0Si5 had the lowest (n=9626, P value=0.211). It is worth mentioning that Ti3Si2 had a high alamar blue reading on day 1 (n= 8492, P value= 0.377) in comparison to the control, however, it did not continue at the same rate until day 7 (n=26761, P value= 0.245). On the other hand, group 2 results showed that both compositions had superior alamar blue readings over group 1 compositions at all three time-points. On day 7, both $50P_2O_5$-25CaO (n= 34855, P value = 0.829) and $45P_2O_5$-30CaO (n= 33547, P value =0.333) had the closest results to control.

Relying on the aforementioned, doping TiO_2 into the glass composition showed to have potential effects on the cellular biocompatibility as previously seen (17). This could be related to the ability of TiO_2 to consolidate the glass structure and eventually to yield in decreasing the rate of degradation and consequently to enhance the cellular metabolic response as was founded in previous studies (20). SiO_2 did not improve the cellular responses when compensated with TiO_2 in group 1 compositions, and hence doping with SiO_2 instead of TiO_2 did not have a positive

effect. However, doping both 5%SiO_2 and 5%TiO_2 into group 2 compositions ($45P_2O_5$-30CaO and $50P_2O_5$-25CaO) had a positive effect in increasing cell number.

4.7.2 Live/Dead Assay

The principle of the Live/Dead assay is that membrane-permeable calcein-AM is cleaved by esterases in live cells to yield cytoplasmic green fluorescence, and membrane-impermeable ethidium homodimer-1 labels nucleic acids of membrane-compromised cells with red fluorescence. Consequently, live and dead cells display green and red fluorescence respectively. All samples showed promising results in terms of keeping the MG63 cells alive after 7 days.

4.7.3 Alkaline Phosphatase Assay

The aim of using this assay was to investigate the ALP values for all compositions. Using different dilutions of the alkaline phosphatase assay kit (SensoLyte® pNPP), an equation was derived to measure the ALP concentration for each composition based on the fluorescence signal obtained.

While on day 7 Ti5Si0, Ti1Si4, Ti3Si2 and Ti4Si1 had significant lower levels of proliferation compared to the other compositions (P value < 0.05); the situation was different on day 14. Ti5Si0, Ti4Si1, Ti3Si2, Ti2Si3 and $45P_2O_5$-30CaO had higher ALP concentration (P value < 0.05) than control group as well as the rest compositions. This significant difference in alkaline phosphatase concentrations between day 7 and day 14 may be due to the fact that the expression of this enzyme is usually highest between day 5 to day 14 during the matrix maturation stage of stem cell differentiation process (21). Although Ti1Si4, Ti0Si5 and $50P_2O_5$-25CaO had

lower values compared to the other compositions in the whole study, their results can also be considered promising as they were similar to control.

Although substituting TiO_2 with SiO_2 did not improve the ability of hMSC's to differentiate into osteoblasts in group 1, it did not cause cytotoxicity. However, doping both 5%SiO_2 and 5%TiO_2 in group 2 compositions had a significantly better effect in 45P_2O_5-30CaO although 50P_2O_5-25CaO composition recorded similar ALP results to the control.

4.7.4 CCK Assay

The aim of using CCK was to count the number of viable cells for all compositions at different time points. Therefore, different known numbers of cells with their CCK readings were used to derive an equation that would help count the number of cells indirectly using the CCK readings obtained.

While on day 1 both Ti5Si0, and Ti3Si2 had higher readings than the rest compositions and similar to the control sample (P value = 0.956 and 0.927 respectively); all samples had similar results on day 4 and 7. On day 7, for example, the control sample had a value of (56973 $\pm$2726) and with respect to it; 50P_2O_5-25CaO had the lowest reading (42189.74 $\pm$ 1432.71) while Ti5Si0 had the highest (48447.42 $\pm$ 2259.43). As the mean differences between the control sample and the highest and the lowest compositions were similar, no negative effect was found of doping SiO_2.

Consequently, compensating TiO_2 with SiO_2 in group 1 was found to give good results. No specific trend was found although compositions with less SiO_2 had insignificant better

readings. Doping both 5%SiO_2 and 5%TiO_2 in group 2 compositions had a better result in 45P_2O_5-30CaO; 50P_2O_5-25CaO having a lower cell numbers.

5. Conclusion

XRD studies confirmed the success of the glass production process, the amorphous nature of all glass samples and indicated that they lacked a crystalline phase. Other results showed that an increase in TiO_2 content directly correlated with an increase in glass density, decrease in degradation, increased trend of Tg and Tm values and Na^+ and Ca^{2+} release in group 1 compositions. Whereas in group 2 compositions, 50P_2O_5-25CaO was less dense than 45P_2O_5-30CaO, it degraded dramatically less, had less Tg and Tm values and released less $Na^+$and Ca^{2+}.

Regarding cell culture studies, live/dead assay showed that all compositions had promising results in terms of keeping the MG63 cells alive after 7 days. However, the rest of the studies showed differences between compositions in terms of cell viability and proliferation.

Within the first group, replacing TiO_2 with SiO_2 did not increase the number of MG63 cells, or the ability of hMSC's cells to differentiate into osteoblasts though it did not cause cytotoxicity. Whereas, within the second group, doping both 5%SiO_2 and 5%TiO_2 into group 2 compositions had a positive effect in increasing the number of MG63 cells in both compositions with 45P_2O_5-30CaO to have better results in terms of cell numbers and improving the ability of hMSC's cells to differentiate into osteoblasts.

Declaration.

The researcher confirms the absence of any conflict of interest.

6. References

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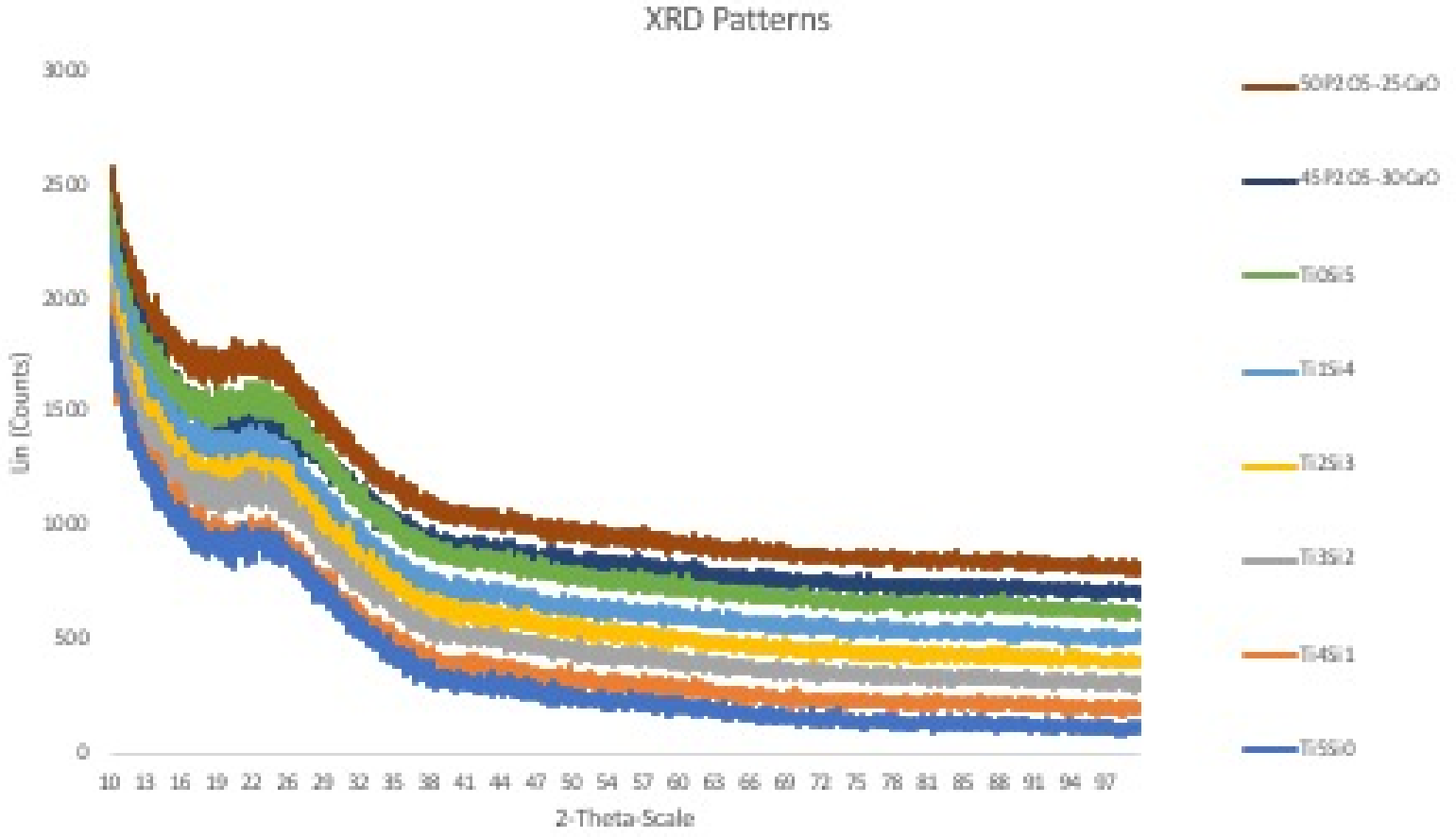
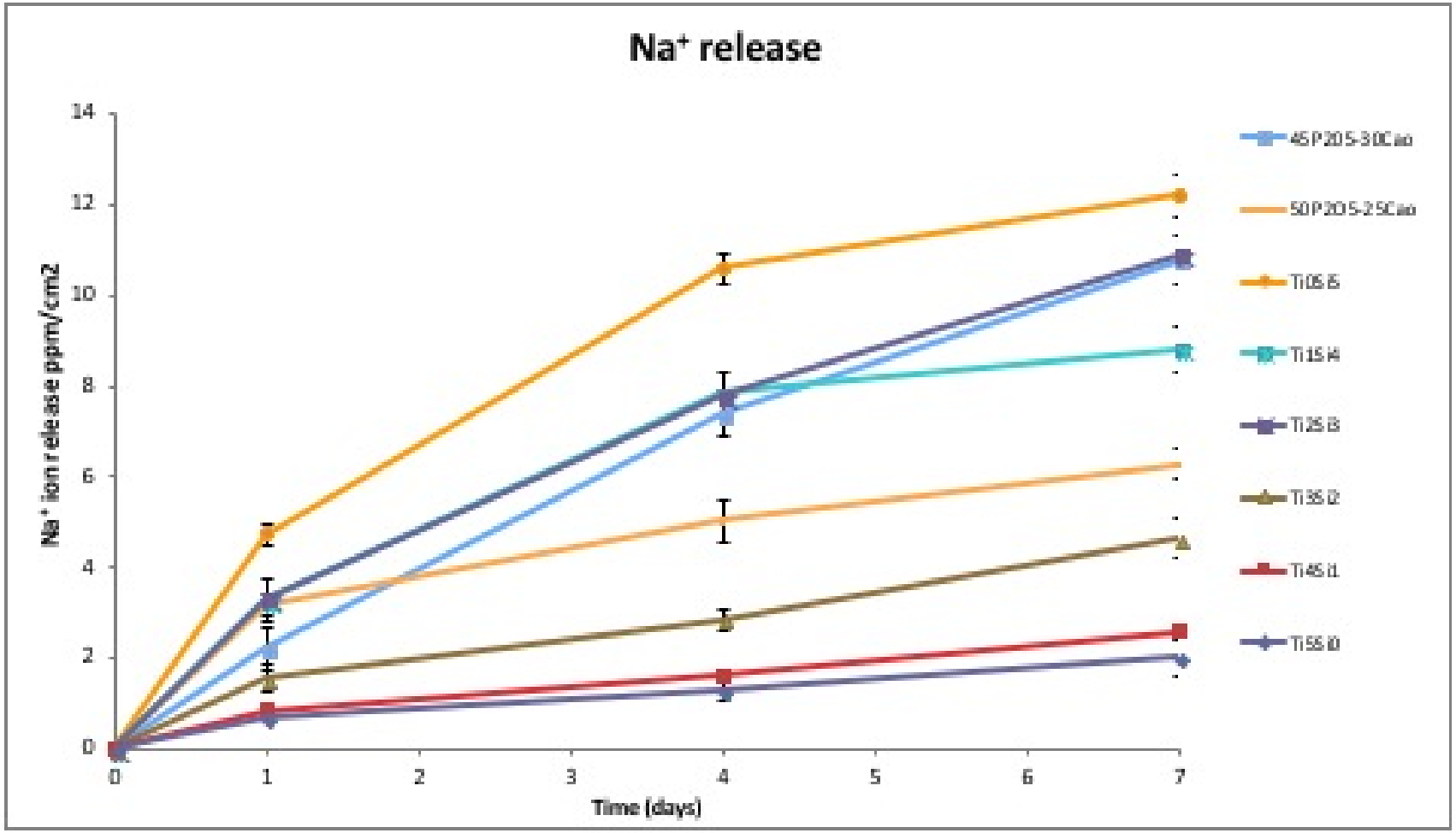
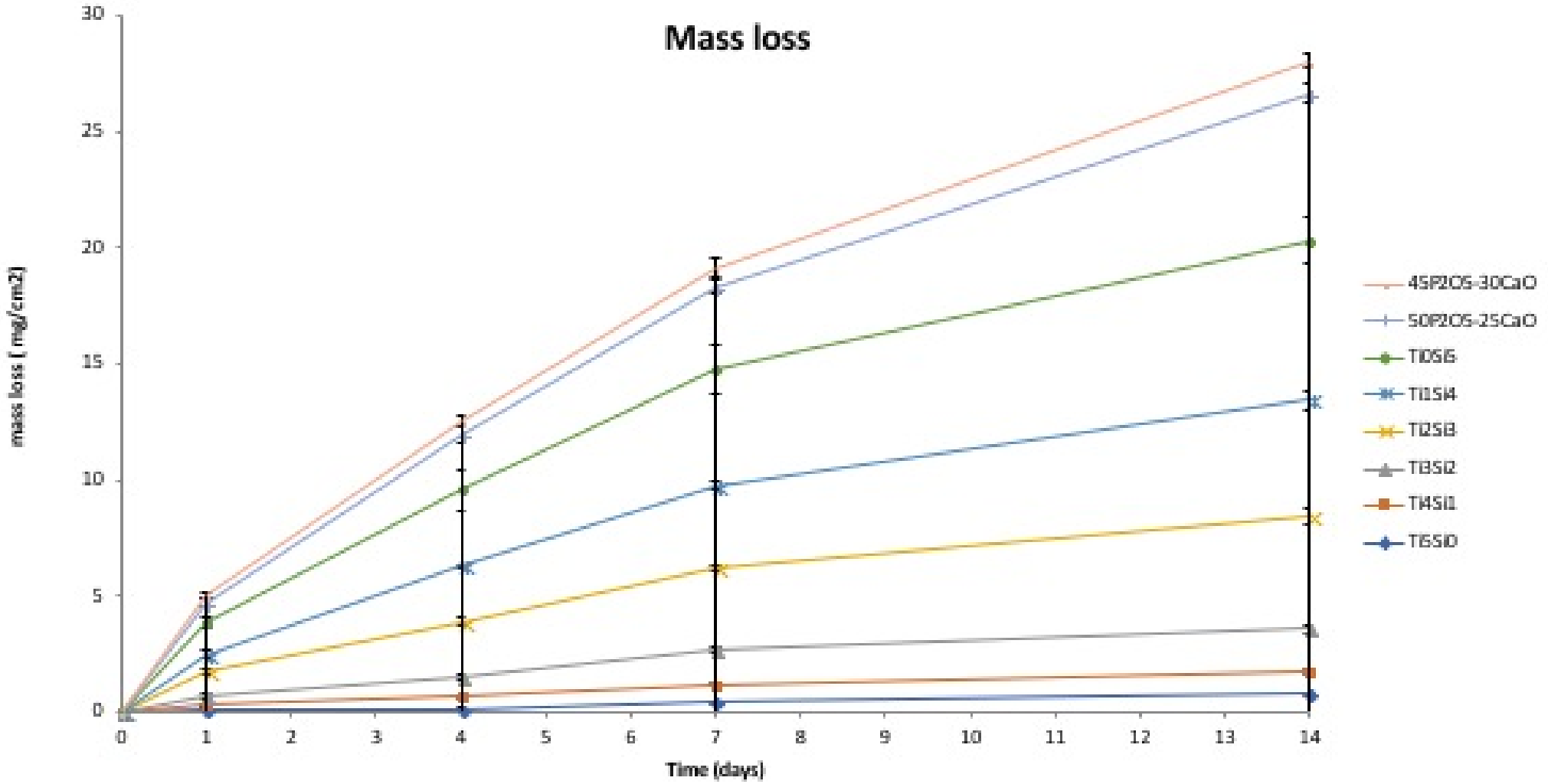
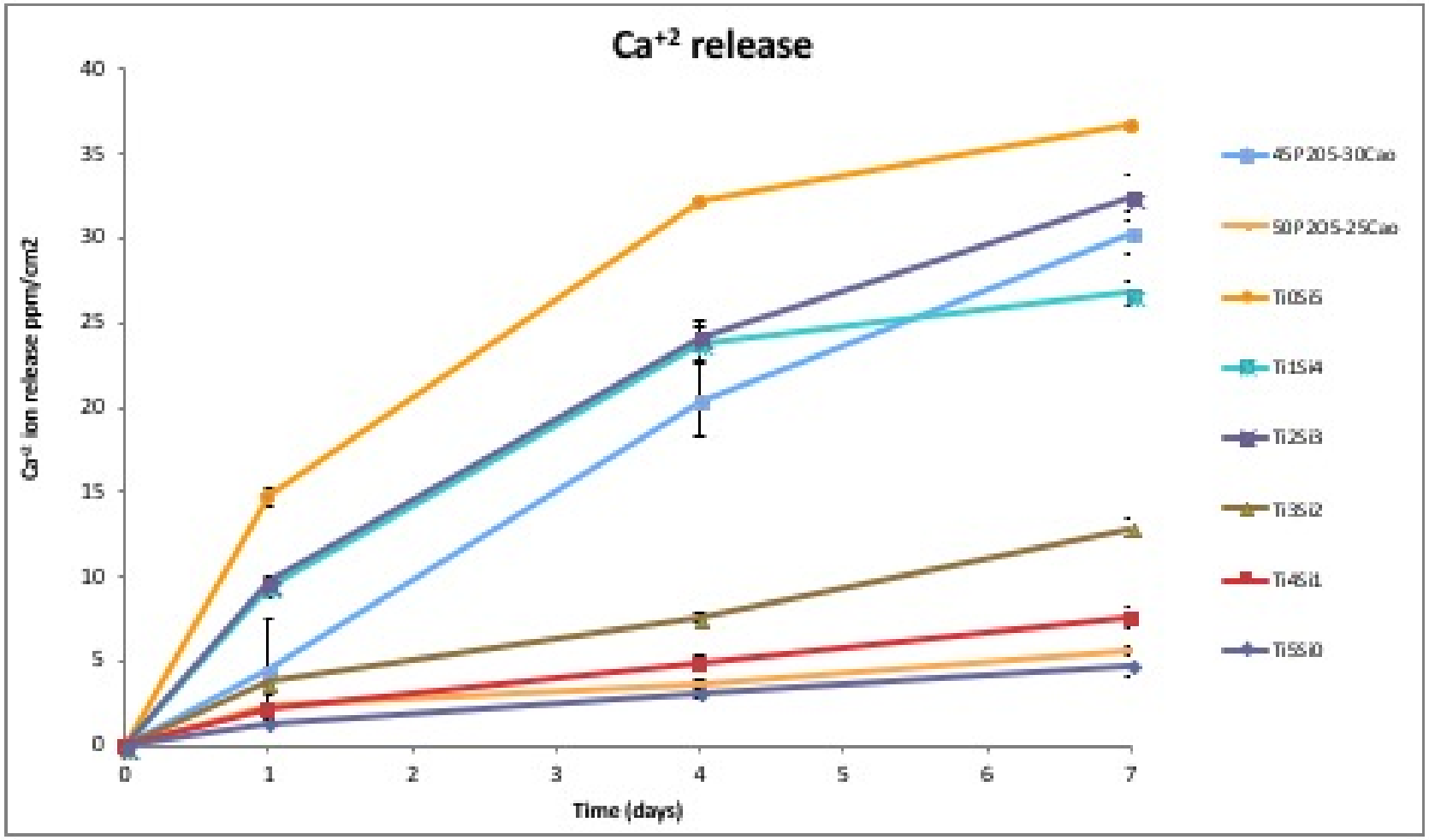
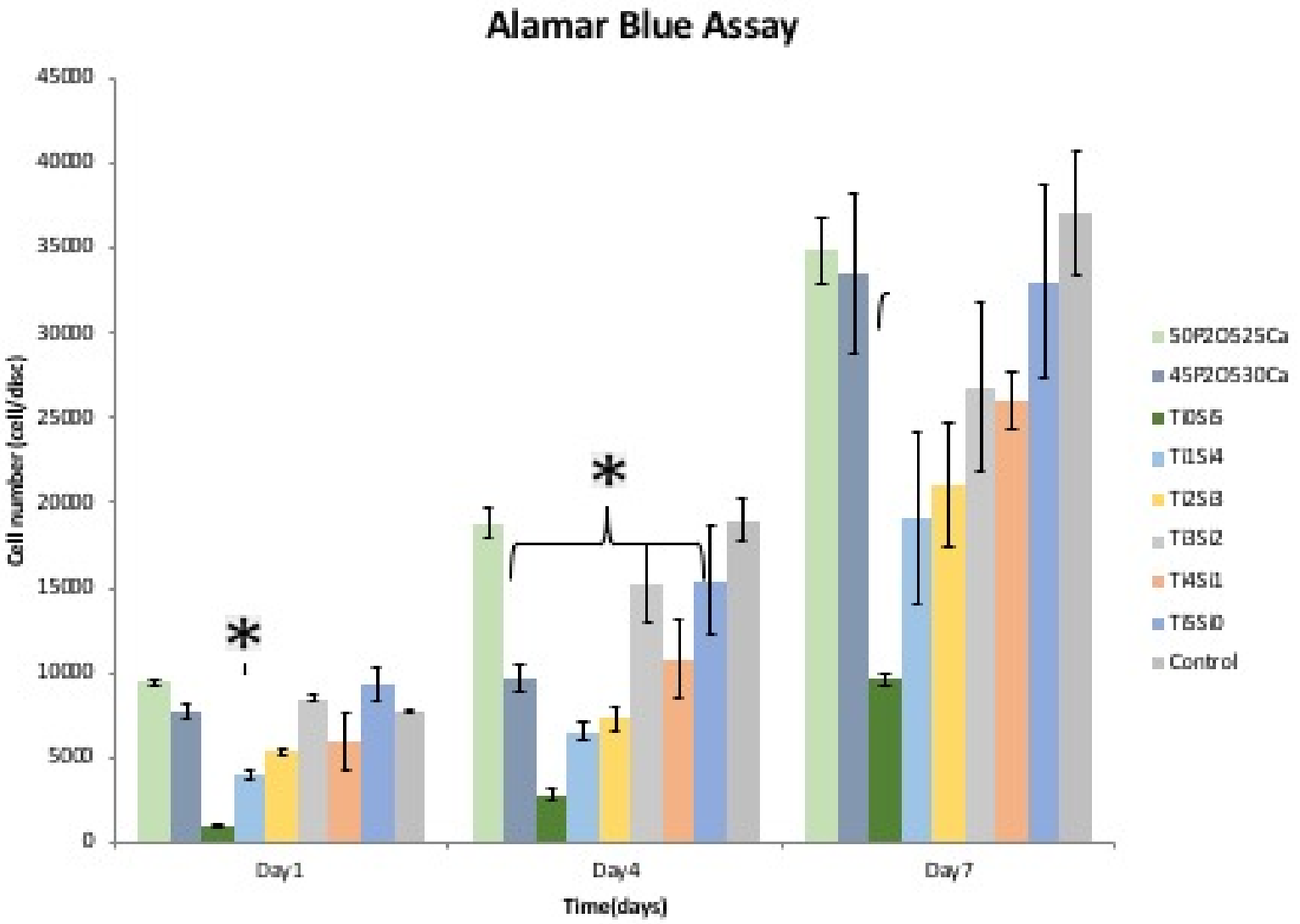

XRD Patterns
30000
25000
20000
15000
10000
5000
0
Lin (Counts)
10 13 16 19 22 26 29 32 35 38 41 44 47 50 54 57 60 63 66 69 72 75 78 81 85 88 91 94 97
2-Theta-Scale
50P2O5-25CaO
45P2O5-30CaO
Ti0S5
Ti1S4
Ti2S3
Ti3S2
Ti4S1
Ti5S0

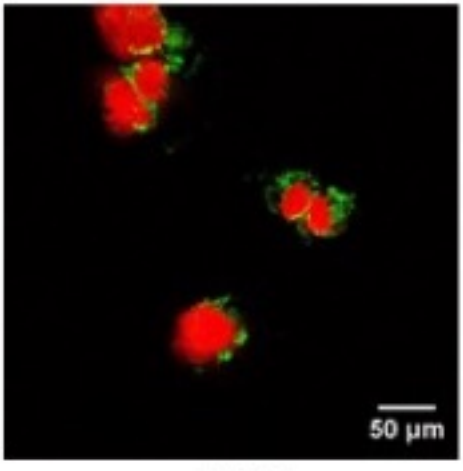

Na+ release
Na+ ion release ppm/cm2
Time (days)
0
2
4
6
8
10
12
14
0
1
2
3
4
5
6
7
45P2O5-30Cao
50P2O5-25Cao
Ti05 6
Ti15 4
Ti25 3
Ti35 2
Ti45 1
Ti55 0


Mass loss
mass loss (mg/cm2)
Time (days)
0
5
10
15
20
25
30
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
45P2O5-30CaO
50P2O5-25CaO
T10S16
T15S14
T25S13
T35S12
T45S11
T65S10

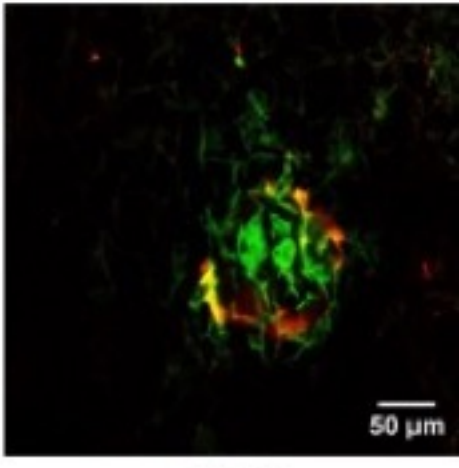

Ca+2 release
Ca+2 ion release ppm/cm2
Time (days)
0
5
10
15
20
25
30
35
40
0
1
2
3
4
5
6
7
45P2O5-30CaO
50P2O5-25CaO
Ti05E
Ti15I4
Ti25I3
Ti35I2
Ti45I1
Ti55I0


Alamar Blue Assay
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Cell number (cell/disc)
*
*
Day1
Day4
Day7
Time(days)
50P2O525Ca
45P2O530Ca
TI0S6
TI1S4
TI2S3
TI3S2
TI4S1
TI5S0
Control

Day 4

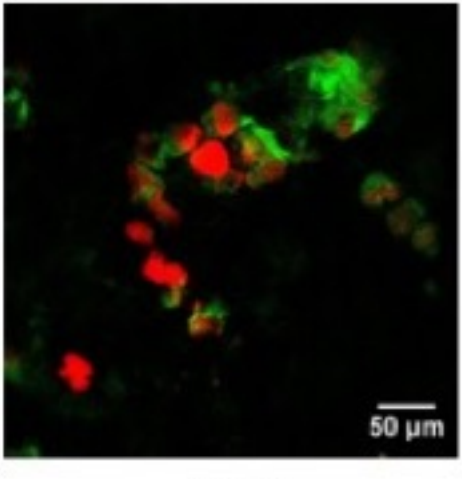


Ti0Si5

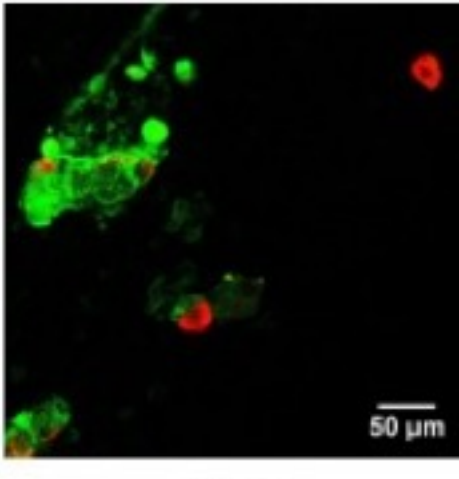


Ti5Si0

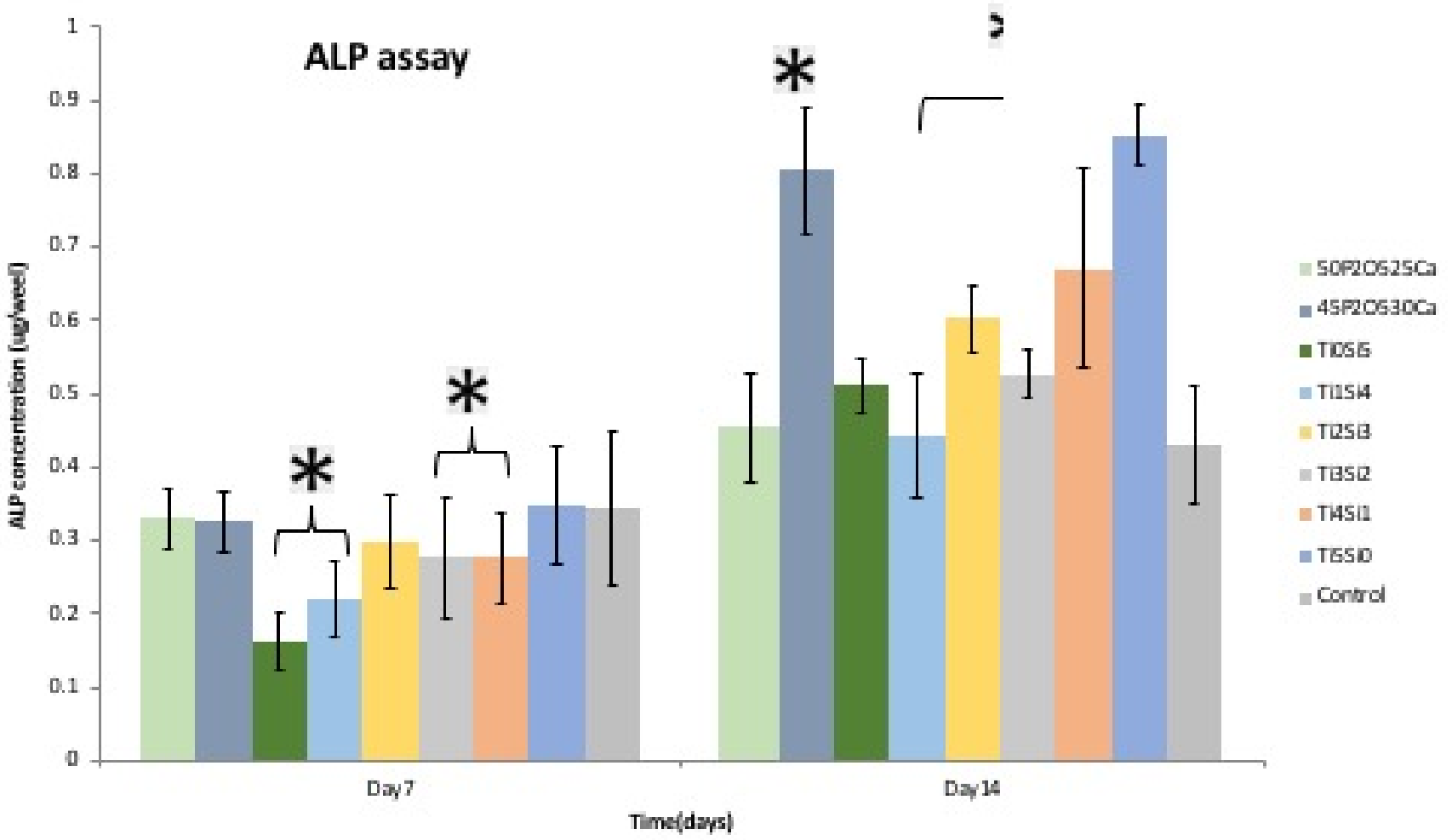
Day 7

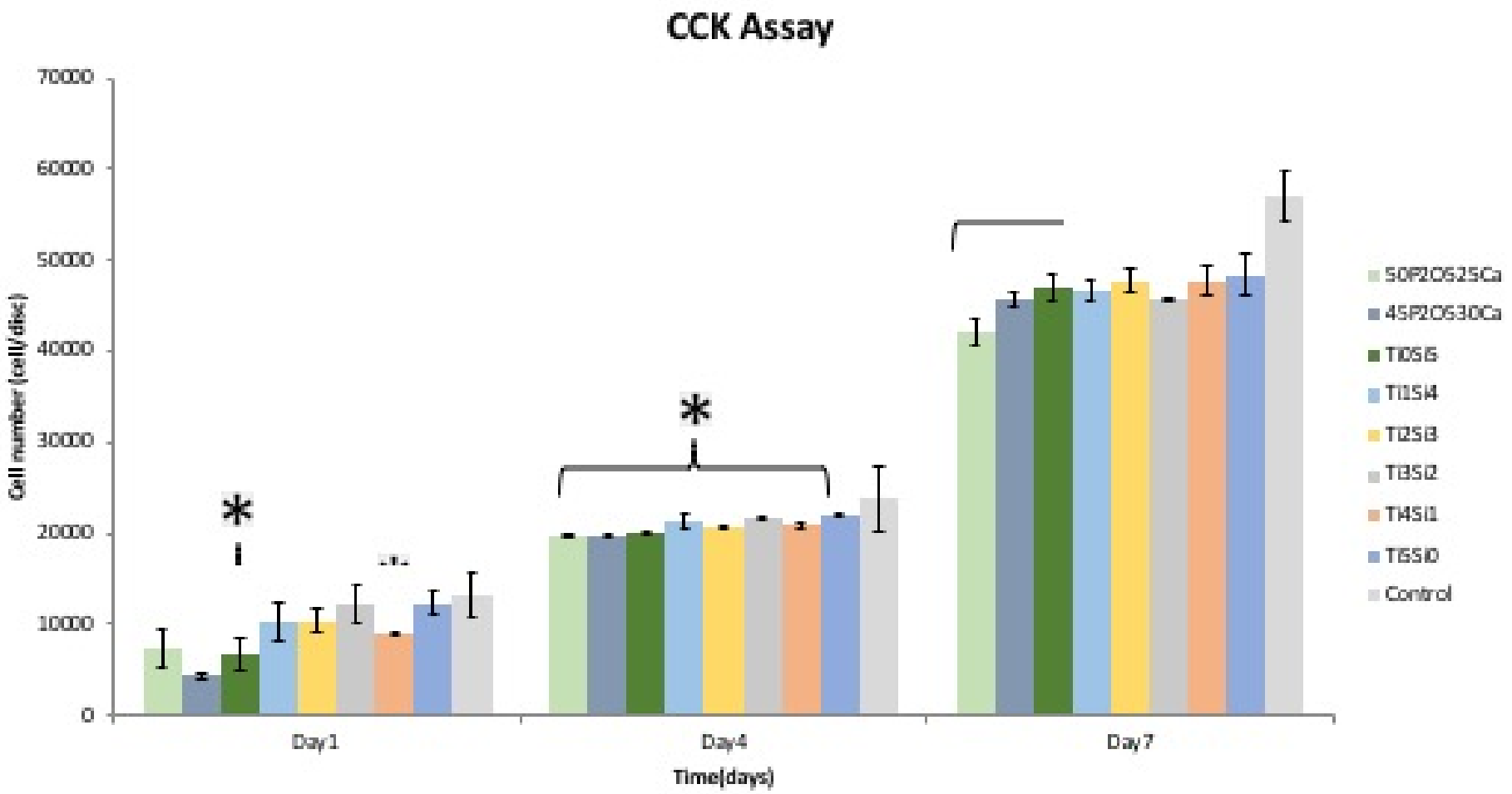


Ti0Si5



Ti5Si0


ALP assay
ALP concentration (ug/well)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Day7
Day14
Time(days)
*
*
*
50P2O525Ca
45P2O530Ca
T10S6
T10S4
T25S3
T35S2
T45S1
T65S0
Control


CCK Assay
70000
60000
50000
40000
30000
20000
10000
0
Cell number (cell/disc)
*
*
Day1
Day4
Day7
Time(days)
50P2O525Ca
45P2O530Ca
T10S6
T11S4
T12S3
T13S2
T14S1
T15S0
Control

Compositions	Code	Glass Compositions (mol%)				
		P_2O_5	CaO	Na_2O	TiO_2	SiO_2
Group 1						
Ti 0% Si 5%	Ti0Si5	50	30	15	0	5
Ti 1% Si 4%	Ti1Si4	50	30	15	1	4
Ti 2% Si 3%	Ti2Si3	50	30	15	2	3
Ti 3% Si 2%	Ti3Si2	50	30	15	3	2
Ti 4% Si 1%	Ti4Si1	50	30	15	4	1
Ti 5% Si 0%	Ti5Si0	50	30	15	5	0
Group 2						
P_2O_5 45%	$45P_2O_5$-30CaO	45	30	15	5	5
Ca 25%	$50P_2O_5$-25CaO	50	25	15	5	5

	ρ glass g/cm^3	Tg °C	Tc °C	Tm °C
Group 1				
Ti0Si5	2.55 ± 0.005	421	681	782
Ti1Si4	2.55 ± 0.003	459	756	823
Ti2Si3	2.56 ± 0.002	466	719	839
Ti3Si2	2.56 ± 0.0003	466	693	844
Ti4Si1	2.59 ± 0.0009	481	733	878
Ti5Si0	2.59 ± 0.002	477	715	867
Group 2				
45P_2O_5-30CaO	2.58 ± 0.016	481	777	846
50P_2O_5-25CaO	2.55 ± 0.003	445	714	778

	Group 1						Group 2		Control
	Ti5Si0	Ti4Si1	Ti3Si2	Ti2Si3	Ti1Si4	Ti0Si5	50P2O5- 25CaO	45P2O5-30CaO	Control
Day1	9.30	9.17	8.50	9.47	8.83	8.37	9.46	9.38	8.38
Day4	9.44	9.54	8.77	9.36	9.54	9.65	9.25	9.74	9.38
Day7	9.21	9.47	8.63	9.18	9.28	9.01	9.44	9.49	8.94