

***In vitro* evaluation of a novel pH neutral calcium phosphosilicate bioactive glass that does not require preconditioning prior to use.**

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**Abstract:**

It is well known that bioactive glasses can cause a significant increase in pH due to the rapid release of calcium and/or sodium ions. Consequently, preconditioning of the glass is usually required prior to surgery to negate the effect of this sudden release of ions. However preconditioning for several days is far from ideal and also preconditioning is not practical for novel organic/inorganic sol-gel hybrids currently

being developed since the organic phase will start to hydrolyze and dissolve. The present study describes a bioactive glass that dissolves without causing a significant change in pH from physiologically optimal values and requires no preconditioning prior to use. The bioactivity of the pH neutral glass, hydroxyapatite formation and cellular responses, are measured and compared directly with results from archetypal 45S5 and S70C30 bioactive glasses. A hydroxyapatite layer was found to rapidly form (within one day) on the surface of the pH neutral glass upon reacting with simulated body fluid. In addition improved cell compatibility was observed compared with 45S5 and S70C30 glasses. Therefore, this pH neutral glass has significant potential for bone repair applications.

## 1. Introduction

Since its discovery by Larry Hench *et al.* in 1969, melt-derived bioactive glass 45S5 (45 wt% SiO<sub>2</sub>, 24.5wt% Na<sub>2</sub>O, 24.5wt% CaO, and 6wt% P<sub>2</sub>O<sub>5</sub>) has been widely used for repairing bone defects and in dental applications [1, 2]. When immersed in body fluid (or simulated body fluid, SBF), these glasses quickly form a layer of hydroxyapatite (HA) on their surfaces [3, 4]. Within the glass calcium and phosphorous aid bioactivity whilst silicon acts as the host. Sodium oxide is present in the glass for two reasons; firstly it aids manufacturing, lowering the melt temperature, and secondly it disrupts the silicate network (i.e. cleaving the strong Si-O-Si bonds) increasing the dissolution of the active ions. Within these glasses sodium has no biological role other than the fact it is relatively inert and can easily be expelled from the body. In parallel to the melt quench derived bioactive glasses, sol-gel derived bioactive glasses have also been widely explored. Although sol-gel derived glasses with the a 45S5 composition have been successfully developed [5] as discussed above there is no biological advantage of incorporating sodium within these glasses. Furthermore, the hydroxyl groups incorporated into the glass during the synthesis essentially perform a similar role, disrupting the silicate network and aiding dissolution. Sol gel derived calcium phospho-silicates have therefore been widely explored e.g. 58S (58 wt% SiO<sub>2</sub>, 33wt% CaO and 9wt% P<sub>2</sub>O<sub>5</sub>), 77S (77wt% SiO<sub>2</sub>, 14wt% CaO and 9wt% P<sub>2</sub>O<sub>5</sub>). However the phosphate content is not essential for bioactivity as phosphorous which is naturally present in the body combines with the released calcium to form apatite. Consequently most sol-gel bioactive glasses have

focused on a simpler calcium silicate S70C30 (70mol% SiO<sub>2</sub> and 30mol% CaO). All these glasses exhibit excellent bioactivity [6, 7] however they typically need preconditioning prior to use.

It is generally accepted that the key step in bioactivity is the ion exchange between Ca and Na ions in the glasses and H<sub>3</sub>O<sup>+</sup> protons in the media, however, this inevitably causes an increase in pH of surrounding media and thus causes cytotoxicity [8-11]. For this reason, preconditioning of bioactive glass samples is usually required before directly seeding cells on them, in order to reduce their cytotoxicity. For example, the S70C30 scaffolds need to be incubated for 3 days before cells were seeded [12]. An *in vivo* study by Swati and co-workers reported that 70S30C bioactive glass preconditioned for 3 days were very effective in regenerating a rat tibia defect, however, without this preconditioning the glasses showed no evidence of osseointegration [11]. 45S5 glass also needs to be preconditioned in physiological fluids for several days to improve their cellular adhesion and osteogenic activity [9]. In real surgeries, preconditioning is not always convenient. In this sense, a bioactive glass with neutral pH would be very beneficial. Furthermore, given that sol-gels have poor mechanical properties there is an increasing drive to develop organic / inorganic sol-gel hybrids [13-15]. It is not possible to pre-soak these hybrids to remove the alkaline ions from the sol-gel phase without beginning to hydrolyze and dissolve the organic components. It is therefore essential that pH neutral sol-gels are formulated.

In principle, increasing the phosphorus content and/or lowering the sodium content should be reduce the pH increasing effect of a bioactive glass. However, in

melt-quenching methods, lowering sodium content will increase the melting temperature and increasing phosphorus content can inhibit the glass forming ability. Furthermore any compositional changes will affect the network connectivity/dissolution and therefore the bioactivity. In conventional sol-gel methods the hydrolysis of alkyl phosphates is very slow and phosphates tend to precipitate instead of gelation[16]. In a previous study, we found that when phytic acid was employed as the phosphorus precursor, much higher phosphorus concentrations could be used in the sol-gel process [17-20], and a much wider range of bioactive compositions could be achieved in the CaO-P<sub>2</sub>O<sub>5</sub>-SiO<sub>2</sub> ternary glass.

Several studies have reported how the gelling process can be controlled by altering the pH through the addition of acids and bases, also several studies have looked at the effect pH of the media has on the dissolution of the glass within the given media. However there has been very little reported on tailoring the glass to dissolve in a pH neutral manner. In theory it should be possible to control the pH by adjusting the sodium, calcium and phosphorus content. However in reality it is challenging to predict the composition required to develop a pH neutral glass due to unknown variables in compositional / structural effects on dissolution rates and precipitation rates. For example the environment of phosphorous (Q<sup>n</sup>) will determine its solubility, whilst sodium ions are much smaller and more mobile than calcium ions and leach much more rapidly causing a rapid rise in pH. Calcium and phosphorous will precipitate thereby removing these ions from the solution.

For the present study a sodium free ternary CaO-P<sub>2</sub>O<sub>5</sub>-SiO<sub>2</sub> glass was selected. It

was assumed that phosphorous would adopt an orthophosphate environment as widely reported for these types of bioactive glasses. It was also assumed that calcium would leach relatively easily from the glass structure. Since precipitation of amorphous calcium phosphate, a likely precursor for the formation of bone mineral, occurs around neutral pH we tailored the glass composition to closely mimic the required Ca/P ratio. Sintered hydroxyapatite,  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ , has a calcium to phosphorous ratio of 1.67 whilst precipitated hydroxyapatite has a less defined composition,  $\text{Ca}_{10-x}(\text{HPO}_4)_x(\text{PO}_4)_{6-x}(\text{OH})_{2-x}$  and a calcium to phosphorous ratio of between 1.33–1.67. For the present study we adopted a  $(\text{CaO})_{0.35}(\text{P}_2\text{O}_5)_{0.108}(\text{SiO}_2)_{0.542}$  composition with a calcium to phosphorous ratio of 1.62 which is close to the ideal composition for sintered HA and lies within the optimal range for precipitate hydroxyapatite.

In the current work we explore if a pH neutral bioactive glass could be achieved by increasing the phosphorus content without reducing the bioactivity. We therefore investigated the *in vitro* performance on a novel pH neutral bioactive glass in the context of bone repair.

## 2. Materials and Methods

### *Materials and preparation*

The following precursors were used without further purification in the sol-gel preparation. Tetraethyl orthosilicate (TEOS,  $\geq 99.0\%$ ) and  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  were

purchased from Sinopharm Chemical Reagent Co., Ltd. Phytic acid (50 wt% aqueous solution) was purchased from Sigma Aldrich. Bioactive glass (45S5) was purchased from Schott in Germany and used as a control glass.

Bioactive glass S70C30 was prepared as previously reported [21]. TEOS (10mL) was hydrolyzed for 30 min in a mixture of ethanol and water at room temperature with 2N HNO<sub>3</sub> as a catalyst. Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O (4.53g) was added gradually whilst continuous stirring. The molar ratio of water to TEOS was 12:1. The obtained clear sol solution was sealed in a polypropylene container and left to gel. The gel was then aged at 60°C for a week, followed by drying at 120°C for a further week and then stabilized at 600°C for 1h to obtain the S70C30 glasses which were ground into fine powders (<38 μm).

Bioactive glass (P<sub>2</sub>O<sub>5</sub>)<sub>0.108</sub>(SiO<sub>2</sub>)<sub>0.542</sub>(CaO)<sub>0.35</sub> (named as PSC\_pH here) was prepared as reported by Li *et al.* [19]. In brief, phytic acid (0.8mL) was firstly mixed with ethanol and water at ambient temperature, then TEOS (2.91mL) was added through a syringe during stirring. After 1 h, Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O powder (1.99g) was added until a transparent solution formed (the sol), which was sealed in polypropylene containers and left to gel. The resultant gel was aged at 60°C for one week and then at 120°C for another two weeks, and then stabilized at 600°C for 1 h to obtain PSC-pH glasses which were also ground into fine powders. The compositions of 45S5, S70C30 and PSC-pH bioactive glasses are summarized in Table 1.

*X-ray diffraction* (XRD) spectra were collected using a Rigaku (D/MAX 2500) instrument with Cu K $\alpha$  radiation ( $\lambda=1.54\text{\AA}$ ), operated at 40 kV and 200 mA. Data was

collected from the surface of pellets with  $2\theta$  values between  $10^\circ$  to  $60^\circ$  and a step size of  $0.02^\circ$ .

*Solid state  $^{31}\text{P}$  NMR* spectroscopy was measured using a Bruker Avance III 400 MHz instrument at a Larmor frequencies of 161.58 MHz. The experiments were conducted at a spinning speed of 12 kHz and referenced using ammonium dihydrogen phosphate ( $\text{NH}_4\text{H}_2\text{PO}_4$ ).

Table 1. Composition of 45S5, S70C30 and PSC-pH bioactive glasses in molar %.

Component	45S5 (mol%)	S70C30 (mol%)	PSC-pH (mol%)
$\text{SiO}_2$	46.1	70	54.2
$\text{CaO}$	26.9	30	35
$\text{P}_2\text{O}_5$	2.6	—	10.8
$\text{Na}_2\text{O}$	24.4	—	—

#### *Bioactivity testing and dissolution measurements*

150 mg of each glass powder (45S5, S70C30 and PSC-pH) was pressed into pellets and immersed in 100 mL of SBF at  $36.5\pm 0.5^\circ\text{C}$  [4, 15, 22, 23]; each sample was measured in triplicate. After immersing in SBF for different intervals the pellets were washed gently with pure water and left to dry in a desiccator without heating. During the course of immersion, ion concentration and media pH were monitored regularly by ICP-MS (Agilent 7900) and a pH meter (PB-10, Sartorius). Apatite formation was evaluated using FTIR, scanning electron microscopy (SEM) and XRD as described above.



*FTIR*: Fourier transform infrared spectra were collected in the range 4000-400 $\text{cm}^{-1}$  on a Bruker Equinox 55 instrument. All samples were diluted with dry KBr, ground into fine powders and pressed into pellets.

*SEM*: The samples were coated with a thin layer of gold (Au) by sputtering (SCD 500). The surface development was observed using a Hitachi (4800) instrument operating at a voltage of 15 kV.

#### *Textural Characterization*

Pore texture of the PSC-pH, 45S5 and S70C30 glasses were analyzed using ASAP 2020 apparatus. Glasses were first degassed in vacuum oven at 200°C for 10 h to remove moisture from the pores and then analyzed with nitrogen adsorption to determine the specific surface area.

#### *Degradation studies*

PSC-pH (150mg) pellets, in triplicates, were immersed in 100 ml of SBF at 36.5 $\pm$ 0.5°C and magnetically stirred at 120 rpm. At different time points (1, 3, 5, 7, 14 and 28d), the glass pellets were removed from their containers and dried under vacuum prior to weighing. To obtain the rate of weight loss, the initial weight ( $M_0$ ) of each sample was measured as well as the weight at time  $t$  ( $M_t$ ) to give a weight loss %:  $\text{weight loss \%} = (M_0 - M_t) / M_0 \times 100\%$ .

#### *Cell evaluation*

*Cell culture*. A preosteoblast cell line (MC3T3-E1; ATCC, CRL-2593, Rockville, MD, USA) was used to investigate the cell compatibility *in vitro*. The MC3T3-E1

cells were cultured in a humidified incubator under an atmosphere containing 5% CO<sub>2</sub> at 37°C. Dulbecco's Modified Eagle Medium ( $\alpha$ -MEM) supplemented with fetal bovine serum (FBS, 10%), penicillin (100 $\mu$ g/mL) and streptomycin (100 $\mu$ g/mL) was used as the culture medium. Cells were sub-cultured every 5 days and maintained at 37°C in a humidified incubator with 5% CO<sub>2</sub>.

*Cell Cytotoxicity and Proliferation.* The glass extracts were prepared as previously shown [24]. 100mg of PSC-pH (or S70C30 / 45S5) bioactive glass, which had been previously sterilized under UV light for 12 h, were soaked in 20mL of  $\alpha$ -MEM preheated to 37 °C. After 24 h at 37°C under sterile conditions,  $\alpha$ -MEM was filtered to obtain glass extracts, which were used as culture medium with 10% fetal bovine serum (FBS).

A Cell Count Kit-8 (CCK-8, Beyotime, Jiangsu, China) was employed to quantitatively evaluate cell viability. Two experimental procedures were carried out to evaluate the cell cytotoxicity and proliferation: (1) MC3T3-E1 was seeded on 96-well culture plates at a density of  $1.7 \times 10^4$  cells/mL, after cell adhesion was verified, the culture medium was replaced by different extracts of glasses (45S5, S70C30 and PSC-pH). Cells incubated in  $\alpha$ -MEM without glass extracts were used as the control. The cells were incubated in the culture plates for 48 h; (2) MC3T3-E1 was seeded on 96-well culture plates at a density of  $1.7 \times 10^4$  cells/mL, after cell adhesion was verified, then 5% of the different glass pellets previously sterilized under UV light for 12 h was added to the culture medium for 2, 4 and 7 d. Then, the CCK-8 solution (20  $\mu$ L per well) was added and incubated for 4 h at 5% CO<sub>2</sub> and 37°C. 100  $\mu$ L of the

reacted reagent from each well was transferred to 96-well plates, and the absorbance at 450 nm was determined using a microplate spectrophotometer (MD, SpectraMax M2, USA). Six parallel replicates of each sample at each time point were measured so that statistical analysis could be performed.

*Cell Morphology.* The glass powders were pressed into discs (~100mg, 13mm in diameter and 0.7mm in thickness). First, the discs were disinfected in 70% ethyl alcohol solution for 2 h, washed twice in sterile phosphate buffer solution (PBS) for 30 min, and then sterilized under high-intensity UV radiation for 4 h. Afterwards, the discs were placed in a 24-well culture plate. MC3T3-E1 cell suspensions (10000 cells per well) were cultured in discs for 1 d. Then, the cell-discs were washed with PBS, and fixed with 2.5% glutaraldehyde at 4°C for 24 h. The cell-discs were dehydrated using ethanol solutions (50, 75 and 95 wt%) and observed by SEM.

#### *Statistical analysis*

The one-way analysis of variance (ANOVA) was used to evaluate significant differences between means in the measured data. Each experiment was repeated at least three times. All quantitative data is presented as mean  $\pm$  standard deviation.

## Results and discussion

### *XRD spectra*

Figure 1 shows the XRD spectra of PSC-pH, 45S5 and S70C30 glasses. As shown by the absence of Bragg peaks all the materials are amorphous. PSC-pH

glasses were tested in comparison to 45S5 and S70C30 glasses because these two glasses have been extensively studied both *in vitro* and *in vivo* [25].

*<sup>31</sup>P NMR.* As shown in Figure 2 the <sup>31</sup>P NMR spectra is dominated by a single peak ~ -2ppm which is assigned to orthophosphate, Q<sup>0</sup>, species. A second feature ~ -8ppm is assigned to Q<sup>1</sup> moieties. Q<sup>0</sup> species account for 90% of the phosphorous environment with the remaining 10% accounted by Q<sup>1</sup> species. This justifies the earlier assumption that phosphorous is loosely incorporated into the structural network and will be readily leached and prevents the pH release caused by the of calcium ions.

#### *Dissolution profiles*

The concentration of ions in the solutions surrounding the glasses 45S5, S70C30 and PSC-pH are presented in Figure 3. Figures 3 a, b and c show the concentrations of silicon, calcium, and phosphorous species which are a summation of the ions released from glasses and the initial concentration of ions present in SBF. The concentration of silicon monotonically increased as a function of time. Si leaches rapid from S70C30 in the first hour, with 45S5 and PSC-pH showing significantly lower concentration of Si. This trend is maintained and silicon concentrations for S70C30, 45S5 and PSC-pH increase from 2.9ppm to approximately 380ppm, 306ppm and 261ppm, respectively in 24 h. Silicon release is a result of the glass dissolution caused by the breaking up of the outer silica network, and has previously been reported to be in the form of monosilicic acid Si(OH)<sub>4</sub> [26]. After the initial rapid release of Si ions from 70S30C and 45S5 during the first hour the gradients become relatively similar for all the

glasses studied.

The calcium concentration increases rapidly during the first few hours (1-3 h) for the 45S5 and 70S30C as ions are released from the glasses, only a very small increase in Ca concentration is observed for the PSC-pH sample in the first few hours. The Ca concentration then gradually decreases as it combined with P and precipitates. The PSC-pH sample has a relatively flat calcium concentration as a function of time implying that either Ca is released relatively slowly from this glass composition or that the high P content binds with the Ca and precipitates as Ca-phosphate.

For 45S5 and S70C30 samples, the concentration of phosphorous decreases sharply with time, and a depletion of calcium ions is also observed between 3-12 h. This is attributed to the precipitation of calcium ions by phosphorous ions from the dissolution media, forming the Ca-P rich layer on the sample surface when the solution is oversaturated. However, for PSC-pH sample, the concentration of phosphorous only decreases in the first 1 h, after which it stays approximately invariable. This is attributed to the constant release of P ions from the PSC-pH glass which contains a high phosphate content.

*pH values* are shown in Figure 4 for each of the samples. It can be seen that the pH of 45S5 and S70C30 increased as a function of time, whereas the pH of PSC-pH was virtually stable. The abrupt increase in calcium concentration in the first few hours (Figure 3c) is similar to the pH increases (Figure 4), supporting the argument that the ion exchange between  $\text{Ca}^{2+}$  ions in the glasses and  $\text{H}_3\text{O}^+$  protons in the SBF media is one of the major reasons for pH increase. The PSC-pH has a higher and

constant phosphorus concentration and helps stabilize the pH, whereas, insufficient phosphorus ions of 45S5 and S70C30 samples cannot precipitate released calcium ions in time, which leads to the increase in pH value. The relatively stable pH is one of the benefits of having a high phosphorus content in the bioactive glass as discussed earlier.

### *Degradation studies*

It has been reported that higher phosphate contents in glasses increases degradation [16]. However, as shown in the above dissolution profiles (Figure 3), PSC-pH glasses with higher phosphate content have lower ion release concentration than other two glasses (45S5 and S70C30). Thus a preliminary evaluation of the PSC-pH glass degradation was conducted by mass loss upon immersion in SBF, as shown in Figure 5. Mass loss of PSC-pH increases in an approximately linearly with the time (as a function:  $y=0.785x$ ), which matches the bone repair process very well. On the contrary, 45S5 glasses have been reported to degrade slowly, taking 1 to 2 years to disappear from the body [27, 28]. The dissolution results further indicate that the lower released ion concentrations in PSC-pH sample is a result of precipitation of calcium ions by phosphate ions rather than lower glass degradation.

### *Morphology of bioactive glass particles*

As shown in Table 2, PSC-pH glass samples have higher BET surface area than other two composition (45S5 and S70C30). Whilst it is known that samples with

higher specific surface areas are generally more reactive upon contacting SBF they also tend to be much more soluble. However in the present study the PSC-pH glass with high phosphate content is found to degrade at a rate suitable for bone repair and regeneration applications, whilst the pH value and released ion concentration increase slowly as a function of time. This means that differences in the ion release rates are not dominated by the specific surface area, otherwise, PSC-pH should have the highest released ion concentrations as well as pH. Again, it suggests that the dominating factor governing the dissolution behaviors of these glass samples is their chemical compositions.

**Table 2** BET surface area of 45S5, S70C30 and PSC-pH glass pellets.

sample	45S5	S70C30	PSC-pH
BET surface area (m <sup>2</sup> /g)	0.19*	31.38	118.91

\*the value was evaluated from average particle size and density because it is too small to be measured by BET apparatus.

#### *Hydroxyapatite formation*

It has been reported that hydroxyapatite forms on the surface of 45S5 and S70C30 within 1d immersion in SBF [7, 9]. As discussed previously we have found that PSC-pH was superior to both 45S5 and 70S30 in terms of the combined release rate of Ca and P which prevents a large shift in pH evolution of surrounding media, furthermore PSC-pH has a larger surface area. Nevertheless, as mentioned earlier, this should not at the expense of reduced hydroxyapatite formation ability (i.e. *in vitro*

bioactivity). Figure 6 shows the XRD and FTIR spectra of the PSC-pH glasses after immersed in SBF for different times. As shown in Figure 6a, the sample before SBF immersion is totally amorphous. Hydroxyapatite (HA) diffraction peaks appear after only 1 d immersion and then increase in magnitude with immersion time, confirming hydroxyapatite formation on the surface of these materials. Monoclinic HA is shown as the reference standard. In the FTIR spectra, the double peaks at 603 and 567 $\text{cm}^{-1}$  are assigned to crystalline phosphate. The FTIR spectra (Figure 6b) shows that before immersion, no phosphate appears for PSC-pH glass, whereas, phosphate signals are detected after only 1 d immersion, which becomes more evident with time, again confirming the formation of apatite.

SEM results also confirm the formation of HA on the surface of the PSC-pH glass after immersion in SBF (Figure 7). It can be seen that both hemispherical and needle like apatite are formed after only 1 d immersion, and the apatite HA layer becomes denser with time. These results are consistent with the XRD and FTIR results, demonstrating the good bioactivity of PSC-pH. The HA formation behavior of PSC-pH is equivalent if not slightly better previously reported 45S5 and S70C30 glasses [7, 9] [29].

#### *Cell viability and morphology*

It is known that cells are sensitive to pH changes and are generally cultured in media with the pH value of 7.2 - 7.4 [8]. Apoptosis or necrosis is likely to be caused by the changes in extracellular pH as the potential across the cell membrane may be altered and ion exchange systems may be inhibited such as  $\text{Na}^+/\text{H}^+$  and  $\text{Ca}^{2+}$ , which



may result in a build-up of intracellular  $H^+$  and denature the proteins. The reported glasses, such as 45S5 and S70C30, drive higher pH alterations because of the release of alkali elements into the tissue culture media [11, 26]. Therefore, significant effort has been devoted to evaluating the effect of preconditioning bioactive glasses on cell response. Preconditioning has been shown to minimize pH change and reduce cell apoptosis, and also to improve cellular adhesion and osteogenic activity [8, 9, 11]. In this work, all the glasses (PSC-pH, 45S5 and S70C30) were used for cell culture without preconditioning. As shown in Figure 8a, at the extracts concentration of 5 mg/mL, 45S5 and S70C30 have significant cytotoxicity, whereas, there is no significant cytotoxicity for the PSC-pH samples. PSC-pH samples also have better cell proliferation comparing to 45S5 and S70C30 samples (Figure 8b), especially at 7d.

Figure 9 shows the morphology of cells adhered on PSC-PH discs. It is found that MC 3T3-E1 cells adhered well on the surface of PSC-pH samples after culturing for 1 d. After culturing for 3 d, MC 3T3-E1 cells show filopodium morphology and spread well on the discs, suggesting that the cells were able to grow and proliferate on the PSC-pH samples without pretreatment. However, there is no cell adhesion found for 45S5 and S70C30 discs (not shown here), in agreement with previous studies. This means that PSC-pH samples have better cell compatibility than the other two typical bioactive glass samples when directly seeded with MC 3T3-E1 cells. Therefore, this new bioactive glass (PSC-pH) has the potential to be used in bone repairing. Further *in vivo* studies are currently being undertaken.

## **Conclusion**

The *in vitro* bioactivity of a new bioactive glass (PSC-pH) with high phosphorus content was explored in this study. It was found upon when PSC-pH glasses were immersed in SBF the pH values of the media were stable and maintained at near the physiological optimal. 45S5 and S70C30 glasses exhibited a significant pH increase as previously reported. The higher phosphorus content in the PSC-pH glass ensured that phosphorous degraded at an appropriate rate such that it could combine with the dissolving calcium allowing them to precipitate into an amorphous calcium phosphate. The PSC-pH glass does not remove the phosphorous ions from the media as quickly and a higher phosphorus ion concentration remains. Consequently the concentration of calcium ions that remained in the media were significantly lower for the PSC-pH glass compared to 45S5 and S70C30. This is because of the rapid formation of HA of PSC-pH (high bioactivity). PSC-pH also had better cell compatibility when directly seeded with cells, where good cell adhesion was observed. On the contrary, no cell adhesion was found for 45S5 and S70C30 under the same cell culture conditions. Therefore, PSC-pH glass is a promising new bone repairing material in terms of biodegradation and biocompatibility. The PSC-pH glass has significant potential as a bioactive glass that does not require preconditioning prior to use and for use in novel organic/inorganic hybrids where it is not possible to precondition the sol-gel phase without disrupting the organic phase.

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## References

- [1] L.L. Hench, Splinter, R. J., Allen, W. C. and Greenlee, T. K., Bonding mechanisms at the interface of ceramic prosthetic materials, *Journal of Biomedical Materials Research Symposium*, 5 (1971) 25.
- [2] L.L. Hench, Chronology of Bioactive Glass Development and Clinical Applications, *New Journal of Glass and Ceramics*, Vol.03No.02 (2013) 7.
- [3] L.L. Hench, The story of Bioglass (R), *Journal of Materials Science-Materials in Medicine*, 17 (2006) 967-978.
- [4] T. Kokubo, H. Takadama, How useful is SBF in predicting in vivo bone bioactivity?, *Biomaterials*, 27 (2006) 2907-2915.
- [5] Q.-Z. Chen, G.A. Thouas, Fabrication and characterization of sol-gel derived 45S5 Bioglass<sup>®</sup>-ceramic scaffolds, *Acta Biomaterialia*, 7 (2011) 3616-3626.
- [6] D. Arcos, M. Vallet-Regi, Sol-gel silica-based biomaterials and bone tissue regeneration, *Acta Biomaterialia*, 6 (2010) 2874-2888.
- [7] P. Saravanapavan, J.R. Jones, S. Verrier, R. Beilby, V.J. Shirliff, L.L. Hench, J.M. Polak, Binary CaO-SiO<sub>2</sub> gel-glasses for biomedical applications, *Bio-Medical Materials and Engineering*, 14 (2004) 467-486.
- [8] J.E. Gough, J.R. Jones, L.L. Hench, Nodule formation and mineralisation of human primary osteoblasts cultured on a porous bioactive glass scaffold, *Biomaterials*, 25 (2004) 2039-2046.
- [9] A.J. Garcia, P. Ducheyne, D. Boettiger, Effect of surface reaction stage on fibronectin-mediated adhesion of osteoblast-like cells to bioactive glass, *Journal of biomedical materials research*, 40 (1998) 48-56.
- [10] A. el-Ghannam, P. Ducheyne, I.M. Shapiro, Bioactive material template for in vitro synthesis of bone, *Journal of biomedical materials research*, 29 (1995) 359-370.
- [11] S. Midha, T.B. Kim, W. van den Bergh, P.D. Lee, J.R. Jones, C.A. Mitchell, Preconditioned 70S30C bioactive glass foams promote osteogenesis in vivo, *Acta Biomaterialia*, 9 (2013) 9169-9182.
- [12] S. Midha, W. van den Bergh, T.B. Kim, P.D. Lee, J.R. Jones, C.A. Mitchell, Bioactive Glass Foam Scaffolds are Remodelled by Osteoclasts and Support the Formation of Mineralized Matrix and Vascular Networks In Vitro, *Advanced Healthcare Materials*, 2 (2013) 490-499.
- [13] O. Mahony, S. Yue, C. Turdean-Ionescu, J.V. Hanna, M.E. Smith, P.D. Lee, J.R. Jones, Silica-gelatin hybrids for tissue regeneration: inter-relationships between the process variables, *Journal of Sol-Gel Science and Technology*, 69 (2014) 288-298.
- [14] G. Poologasundarampillai, B. Yu, O. Tsigkou, D. Wang, F. Romer, V. Bhakhri, F. Giuliani, M.M. Stevens, D.S. McPhail, M.E. Smith, J.V. Hanna, J.R. Jones, Poly( $\gamma$ -glutamic acid)/silica hybrids with calcium incorporated in the silica network by use of a calcium alkoxide precursor, *Chemistry*, 20 (2014)

8149-8160.

- [15] A.L. Li, H. Shen, H.H. Ren, C. Wang, D.C. Wu, R.A. Martin, D. Qiu, Bioactive organic/inorganic hybrids with improved mechanical performance, *J. Mat. Chem. B*, 3 (2015) 1379-1390.
- [16] E.A. Abou Neel, D.M. Pickup, S.P. Valappil, R.J. Newport, J.C. Knowles, Bioactive functional materials: a perspective on phosphate-based glasses, *Journal of Materials Chemistry*, 19 (2009) 690-701.
- [17] D. Qiu, P. Guerry, J.C. Knowles, M.E. Smith, R.J. Newport, Formation of functional phosphosilicate gels from phytic acid and tetraethyl orthosilicate, *Journal of Sol-Gel Science and Technology*, 48 (2008) 378-383.
- [18] A.L. Li, Y.P. Ma, D. Qiu, Formation of six-coordinated silicon in calcium phosphosilicate xerogels assisted by polyols at low temperature and pressure, *Chinese Chemical Letters*, 26 (2015) 768-772.
- [19] A.L. Li, D. Qiu, Phytic acid derived bioactive CaO-P2O5-SiO2 gel-glasses, *Journal of Materials Science-Materials in Medicine*, 22 (2011) 2685-2691.
- [20] A. Li, D. Wang, J. Xiang, R.J. Newport, M.X. Reinholdt, P.H. Mutin, D. Vantelon, C. Bonhomme, M.E. Smith, D. Laurencin, D. Qiu, Insights into new calcium phosphosilicate xerogels using an advanced characterization methodology, *Journal of Non-Crystalline Solids*, 357 (2011) 3548-3555.
- [21] Y.S. Sun, A.L. Li, H.H. Ren, X.P. Zhang, C. Wang, D. Qiu, Removal of residual nitrate ion from bioactive calcium silicate through soaking, *Chinese Chemical Letters*, 27 (2016) 579-582.
- [22] C. Wang, Y. Xie, A. Li, H. Shen, D. Wu, D. Qiu, Bioactive Nanoparticle through Postmodification of Colloidal Silica, *Acs Applied Materials & Interfaces*, 6 (2014) 4935-4939.
- [23] C. Wang, H. Shen, Y. Tian, Y. Xie, A.L. Li, L.J. Ji, Z.W. Niu, D.C. Wu, D. Qiu, Bioactive Nanoparticle-Gelatin Composite Scaffold with Mechanical Performance Comparable to Cancellous Bones, *Acs Applied Materials & Interfaces*, 6 (2014) 13061-13068.
- [24] M. Alcaide, P. Portoles, A. Lopez-Noriega, D. Arcos, M. Vallet-Regi, M.T. Portoles, Interaction of an ordered mesoporous bioactive glass with osteoblasts, fibroblasts and lymphocytes, demonstrating its biocompatibility as a potential bone graft material, *Acta Biomaterialia*, 6 (2010) 892-899.
- [25] J.R. Jones, Review of bioactive glass: From Hench to hybrids, *Acta Biomaterialia*, 9 (2013) 4457-4486.
- [26] P. Sepulveda, J.R. Jones, L.L. Hench, In vitro dissolution of melt-derived 45S5 and sol-gel derived 58S bioactive glasses, *Journal of biomedical materials research*, 61 (2002) 301-311.
- [27] E.S. Tadjoeidin, G.L. de Lange, P.J. Holzmann, L. Kuiper, E.H. Burger, Histological observations on biopsies harvested following sinus floor elevation using a bioactive glass material of narrow size range, *Clinical Oral Implants Research*, 11 (2000) 334-344.
- [28] E.S. Tadjoeidin, G.L. de Lange, D.M. Lyaruu, L. Kuiper, E.H. Burger, High concentrations of bioactive glass material (BioGran (R)) vs. autogenous bone for sinus floor elevation - Histomorphometrical observations on three split mouth clinical cases, *Clinical Oral Implants Research*, 13 (2002) 428-436.
- [29] L.L. Hench, Bioceramics - from Concept to Clinic, *Journal of the American Ceramic Society*, 74 (1991) 1487-1510.

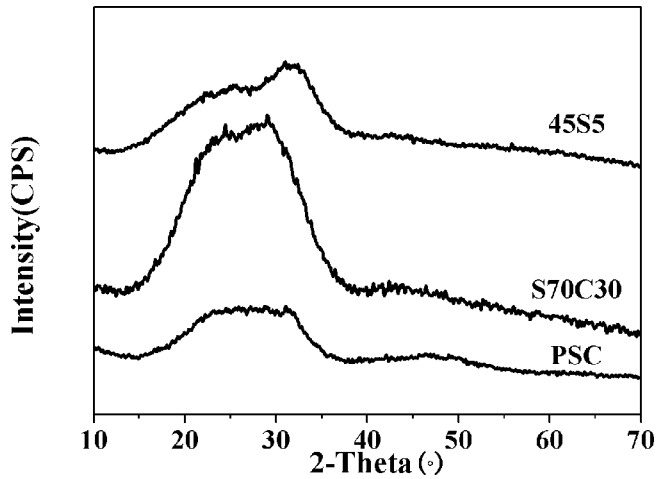


Figure 1. XRD spectra of PSC-pH, 45S5 and S70C30 glasses. The datasets are offset for clarity.

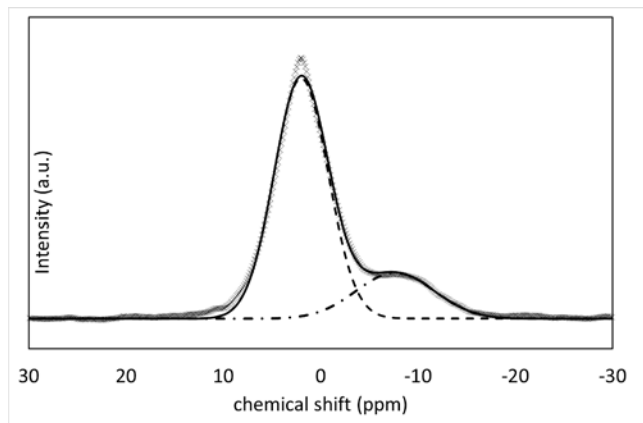


Figure 2.  $^{31}\text{P}$  NMR. The experimental data is given by the crosses, Gaussian fits are given by the broken curves and the solid curve is the total fit.

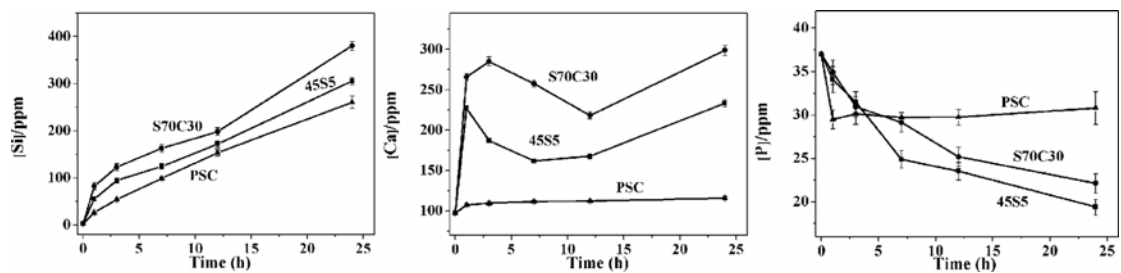


Figure 3. Concentration of Si, Ca and P ions in the surrounding media as determined by ICP are given in Figures 3(a), (b) and (c) respectively for 45S5, S70C30 and PSC-pH in SBF.

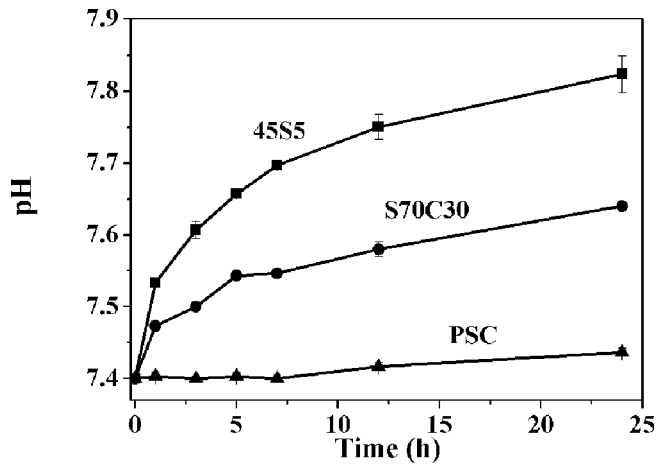


Figure 4. pH values of PSC-pH, 45S5 and S70C30 glasses as a function of time.

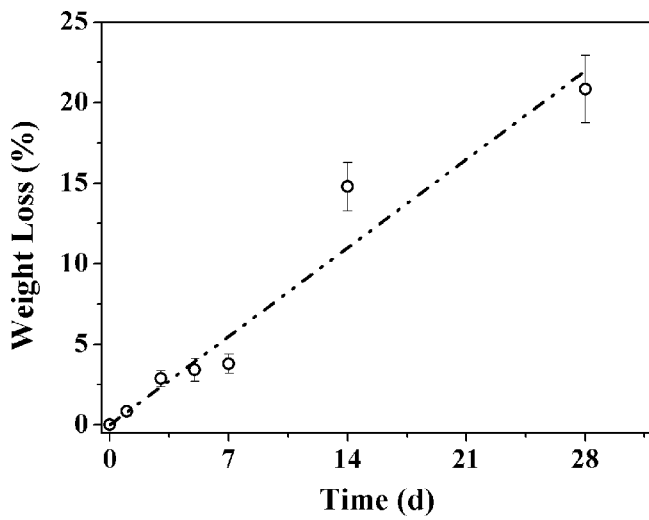


Figure 5. Weight loss in SBF solution as a function of soaking time for PSC-pH glass.

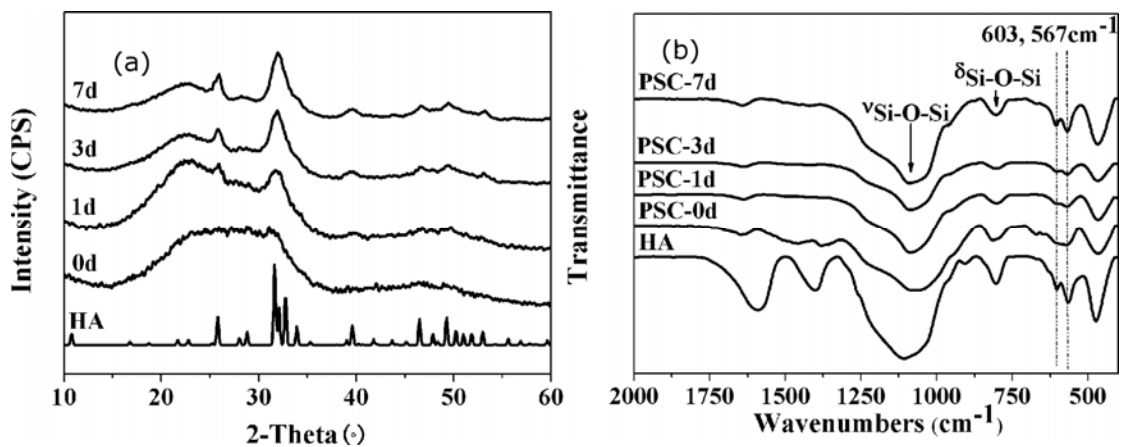


Figure 6. XRD (a) and FTIR (b) spectra of PSC-pH glasses after immersing in SBF for different times (0, 1, 3 and 7 d).

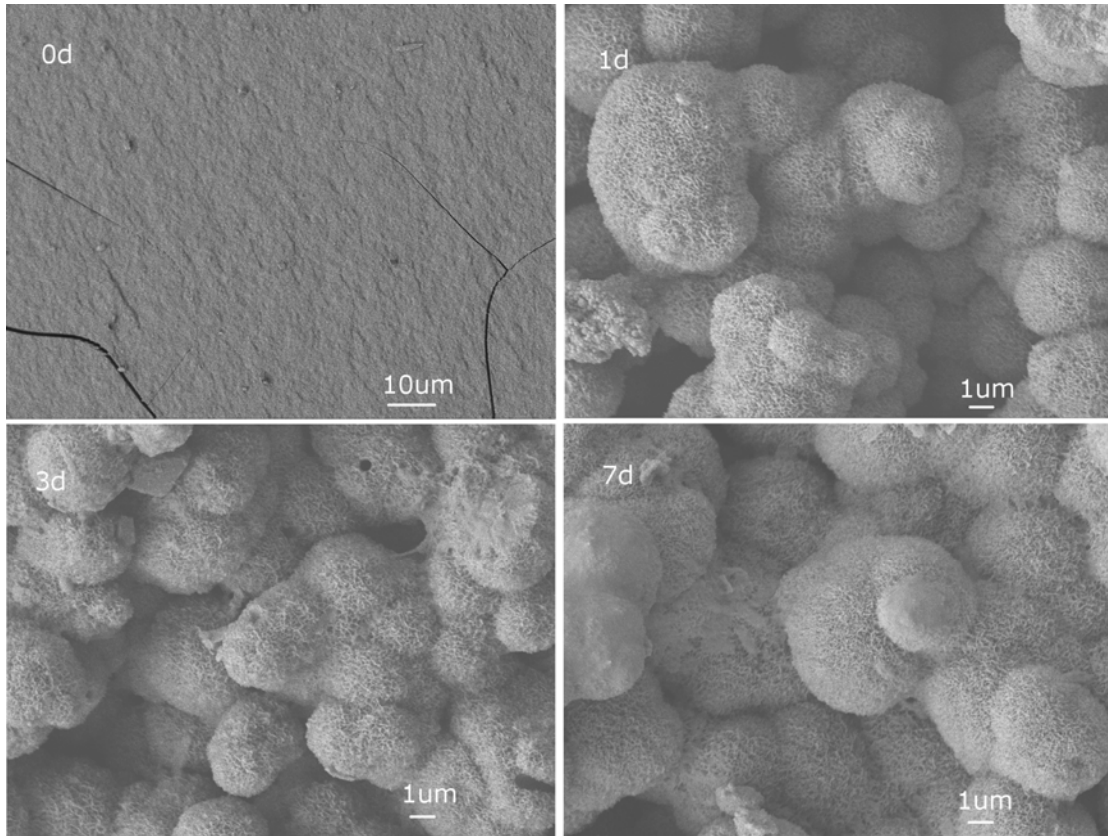


Figure 7. SEM images of PSC-pH glass after immersion in SBF for different time periods (0, 1, 3, 7 d).

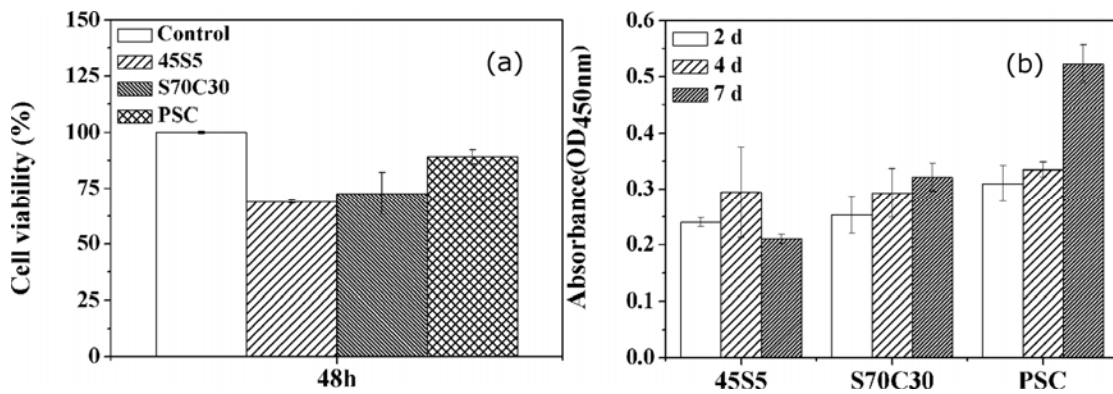


Figure 8. CCK-8 assay for MC 3T3-E1 cells cultured (a) in the absence (control) or in the presence of different glass (45S5, S70C30 and PSC-pH) extracts (5mg/mL) for 48 h (b) with 5% of 45S5, S70C30 and PSC-pH glass pellets for different times (2, 4 and 7d) (statistical significance: \*\*\* $p < 0.005$ , \*\* $p < 0.01$ ).

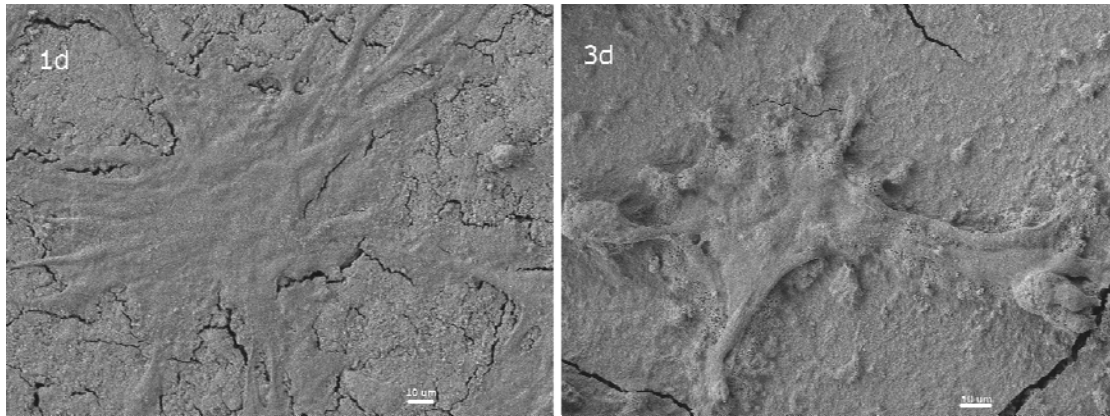


Figure 9. SEM images of preosteoblast MC 3T3-E1 cells cultured on PSC-pH discs for 1d and 3d.