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Dissolution behavior and cell compatibility of alkali-free MgO-CaO-SrO-TiO_2-P_2O_5 glasses for biomedical applications

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Abstract: Owing to their controlled solubility, phosphate invert glasses are of interest for use as temporary implant materials or tissue engineering scaffolds for controlled ion release. MgO-CaO-SrO-TiO_2-P_2O_5 invert glasses were prepared and their dissolution behavior and cell response were examined. MgO addition to the phosphate invert glass system improved glass formation, owing to the relatively large field strength of Mg^{2+} ions. In osteoblast-like MC3T3-E1 cell culture tests, cell numbers on the invert glasses were significantly larger compared with the control, possibly caused by the release of Mg^{2+} ions promoting enhanced cell adhesion and proliferation. Alkaline phosphatase (ALP) activity varied with glass composition, with higher strontium for calcium substitution (33 to 100%) showing highest ALP activity. This effect may be caused by the release of strontium ions from the glasses.

Keywords: Phosphate glass; magnesium; calcium; strontium; ion release; cell response; MC3T3-E1

1 Introduction

In first- and second-generation biomaterials, the main focus was their biocompatibility and bioactivity/bioresorbability, respectively [1]. Nowadays, much attention has been paid to gene activation materials. Certain ions, such as silicate [2, 3], magnesium (Mg^{2+}) [4–7] and strontium (Sr^{2+}) [8–10], have been reported to be effective for gene activation. Mg^{2+} ions have been reported to accelerate osteoblast adhesion, enhance cell proliferation and differentiation [4–7], while Sr^{2+} ions have a dual effect in the stimulation of osteoblast differentiation and inhibition of osteoclast replication and differentiation [8–10].

Calcium phosphate invert glasses, which consist of small phosphate groups such as ortho- and pyrophosphates, have been previously characterized by our group [11]. They showed *in vitro* hydroxyapatite formation (bioactivity) in simulated body fluid (SBF) [12], and when used as a coating on a Ti-29Nb-13Ta-4.6Zr alloy, the coated alloy directly bonded with bone in animal tests [13]. In another work, calcium/strontium-containing phosphate invert glasses were shown to continuously release Sr^{2+} ions [14], which may enhance the activity of osteoblasts and inhibit that of osteoclasts [15]. However, their glass-forming ability decreased with increasing SrO content, while phosphate invert glasses are known for their pronounced tendency to crystallize during heat treatment [16]. MgO-containing phosphate invert glasses, by contrast, showed excellent glass-forming ability in our previous work [17, 18], and Mg^{2+} ions have been suggested to improve glass formation in phosphate glasses [19].

Therefore, we prepared phosphate invert glasses containing both Mg^{2+} ions, to improve glass formation and enhance cell adhesion, and Sr^{2+} ions, to stimulate osteoblasts and inhibit osteoclasts, and investigated their ion release behavior and cytocompatibility *in vitro*.

2 Methods

Glasses with compositions of 7MgO·(60-*x*)CaO·*x*SrO·30 P_2O_5·3TiO_2 (mol%; *x* = 0 to 60, nominal compositions, *cf.* also Table 1; denoted by *y*Sr; where *y* indicates the percentage of SrO for CaO substitution from 0 to 100%) were prepared using MgO (99%), $CaCO_3$ (99.5%), $SrCO_3$ (98%), H_3PO_4 (85% solution) and TiO_2 (99.5%) (all from Kishida Chemical Co., Japan). The reagents were mixed with dis-

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Table 1: Glass composition (mol%) and Sr for Ca substitution (%).

	P_2O_5	CaO	SrO	MgO	TiO_2	Sr for Ca Substitution
0Sr	30	60	0	7	3	0
17Sr	30	50	10	7	3	17
33Sr	30	40	20	7	3	33
50Sr	30	30	30	7	3	50
67Sr	30	20	40	7	3	67
83Sr	30	10	50	7	3	83
100Sr	30	0	60	7	3	100

tilled water to make a slurry, which was dried under an infrared lamp overnight and stored at 140°C. The mixture was melted in a Pt crucible at 1400°C for 30 min and then quenched between stainless steel plates at room temperature to prevent crystallization.

Glass transition (T_g) and crystallization temperature (T_c, defined as the onset of crystallization) of the glasses were obtained from differential thermal analysis (DTA; heating rate 5 K/min; Thermo plus TG8120, Rigaku Co., Japan). Glass structure was investigated by laser Raman spectroscopy (NRS-2000, 514.4 nm, JASCO Co., Japan).

Glass samples were pulverized and sieved to obtain a particle size fraction between 125 and 300 μm. Tris buffer solution was prepared by dissolving 6.118 g of tris(hydroxymethyl)aminomethane ($NH_2C(CH_2OH)_3$, Kishida Chemical Co., Japan) in 1 L of distilled water at 37°C, and adjusting pH to 7.4 at 37°C using 1 N hydrochloric acid. Fifteen mg of the glass powders were soaked in 15 mL of Tris buffer solution at 37°C for 7 days. The concentrations of Mg^{2+}, Ca^{2+}, Sr^{2+}, P^{5+} and Ti^{4+} ions in Tris buffer solution were measured using inductively coupled plasma atomic emission spectroscopy (ICP-AES, ICPS-7000, Shimadzu Co., Japan).

Glass pellets (powder compacts) were prepared for cell culture tests. The glass powders were pulverized using a planetary ball mill (Planetary Mono Mill Pulverisette 6, Fritsch GmbH, Japan) for 30 min at 400 rpm, with zirconia balls of 3 mm-diameter and methanol. After milling, the slurries were dried in an oven at 180°C for 3 h. Two hundreds μg of glass powder was put into 15 mm-diameter molds and pressed at 350 MPa for 5 min, to obtain a powder compact of 0.6 mm thickness. The resulting compact was heat-treated at 600°C for 12 h. The amorphous state of the glasses was examined by X-ray diffraction (XRD, RAD-B, Rigaku Co., Japan). For cell culture tests, glass powder compacts were dry-sterilized at 180°C for 90 min, and 10% fetal bovine serum (GIBCO™, Life Technologies, Japan)-containing α-minimum essential medium (α-MEM with L-glutamine and phenol red; Wako Chemical Co., Japan) was used. Sterilized powder compacts were placed into 24-well plates (n = 3). Mouse osteoblast-like cells (MC3T3-E1 cells) were seeded by adding 1 mL of culture medium containing cells at a concentration of 2×10^4 mL^{-1}. The culture medium was replaced after 1 day and subsequently after every 2 days of culture. Cell culture was performed at 37°C in 5% CO_2 atmosphere. After 3 to 10 days of culture, cell numbers on the glass powder compacts were measured using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan). After a predefined period of culture, the medium was replaced and 100 μL of CCK-8 reagent was added. After 2 h of incubation (37°C, 5% CO_2), the absorbance at 450 nm was evaluated using a microplate reader (Sunrise REMOTE, TECAN Co., Japan). Cell numbers were calculated from a standard curve obtained using cell concentrations in the range of 0 to 25×10^4 mL^{-1}; tissue culture plastic plates (Thermanox™, NUNC™, Thermo Scientific, Japan) were used as a control. Alkaline phosphatase (ALP) activity, a marker of osteoblast differentiation and mineralization, was evaluated in MC3T3-E1 cells cultured on powder compacts, as described above using the following method. For this experiments, 0Sr was used for the control without the effect of Sr^{2+} ions. Phosphate buffered saline (PBS) was prepared with NaCl (8 g), KCl (0.2 g), Na_2HPO_4 (1.15 g) and KH_2PO_4 (0.2 g) dissolved in 1 L of sterile filtered ultra-pure water (milli-Q®), with pH 7.4 at 37°C. The samples were washed with PBS, and 200 μl of p-Nitrophenyl phosphate reagent (SIGMA FAST™, Sigma-Aldrich, Japan) was then added to each well and incubated for 6 min at 37°C and 5% CO_2. After incubation, 60 μL of reaction solution was transferred to a 96-well plate and 60 μL of 0.2 N NaOH was added to stop further reaction. 100 μL of the solution was transferred to a new well and the absorbance at 405 nm was measured with a microplate reader. ALP levels were calculated from a standard curve, in the range of 1.5 to 50 μmol/L.

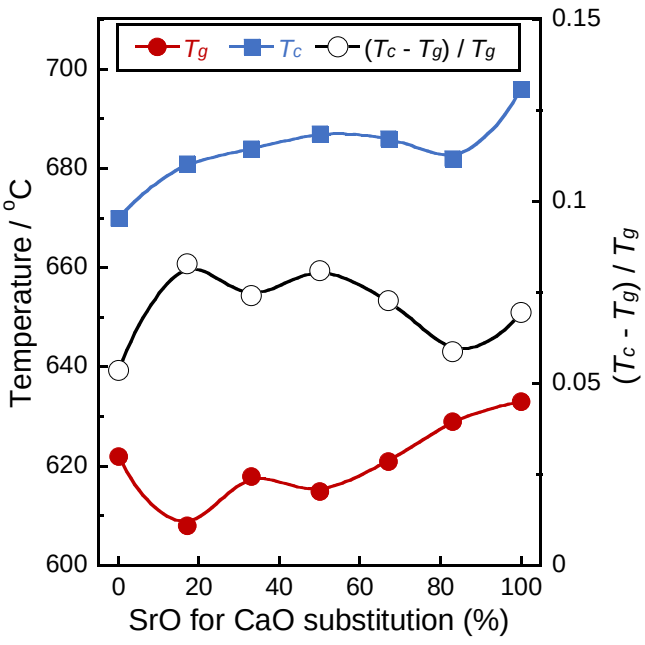

Figure 1: Glass transition (T_g) and crystallization (T_c) temperatures and glassification degree (($T_c - T_g$) / T_g) of the *y*Sr glasses.

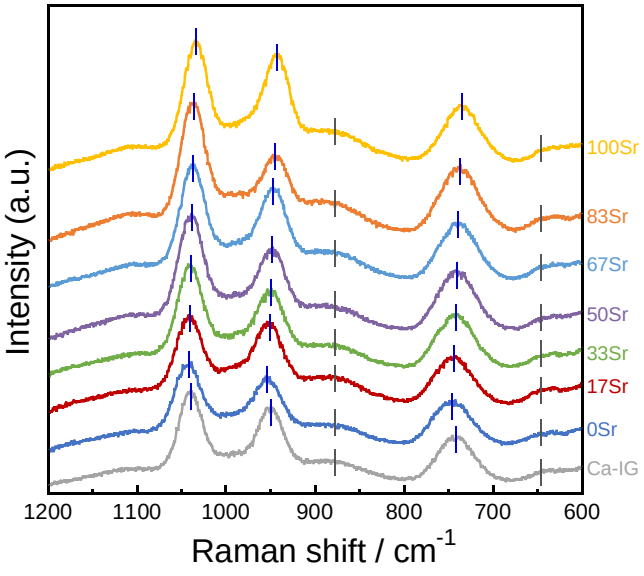

Figure 2: Raman spectra of the *y*Sr glasses.

3 Results

T_g varied between 608 and 633°C and T_c varied between 670 and 696°C (Fig. 1). The glassification degree, defined as $(T_c - T_g)/T_g$ [20] and used as an indicator of glass forming ability, varied between 0.05 to 0.08, with the mixed Ca/Sr glasses having the highest values (Fig. 1).

The glasses show Raman bands corresponding to Q^0 and Q^1 (ortho- and pyro-phosphate) groups (Fig. 2), including the POP_{sym} stretching mode of bridging oxygen in Q^1 (750 cm^{-1}), the $(PO_4)_{sym}$ stretching mode of non-bridging oxygen in Q^0 (950 cm^{-1}) and the $(PO_3)_{sym}$ stretching vibration of non-bridging oxygen in Q^1 species (1040 cm^{-1}) [21]. The wavenumbers at the maxima of Q^0 and Q^1 bands in 0Sr blue-shifted, from 950 cm^{-1} to 955 cm^{-1} and 1040 cm^{-1} to 1043 cm^{-1}, respectively, which compared to a similar, previously worked composition containing Na_2O rather than MgO [14] (shown in Fig. 2 as Ca-IG). With increasing SrO for CaO substitution, the maxima of Q^0 and Q^1 bands red-shifted, from 955 cm^{-1} to 944 cm^{-1} and 1043 cm^{-1} to 1034 cm^{-1}, respectively. In contrast, the bands corresponding to Ti-O stretching vibration of TiO_6 octahedra (650 cm^{-1}) and Ti-O stretching vibration of TiO_4 tetrahedra (880 cm^{-1}) showed no significant change in position with increasing SrO for CaO substitution [22].

Figure 3 shows ion amounts dissolved and released from the glasses into Tris buffer solution. Mg^{2+}, P^{5+} and Ti^{4+} ions amounts dissolved from the glasses with mixed CaO/SrO compositions were smaller than these of 0Sr and 100Sr. The amount of Ca^{2+} and Sr^{2+} ions in Tris buffer solution decrease and increase with increasing SrO substitution, respectively. Sr^{2+} ion concentrations (in mmol L^{-1}) dissolved from the glasses are shown in Fig. 3(c), which reveals that the dissolution of Sr^{2+} ions increased gradually with increasing soaking time and with increasing SrO substitution. Dissolution behavior of the glasses may be influenced by the molar weight of the glasses, which increase with increasing SrO substitution. Figure 3(f) shows the sum of Ca^{2+} and Sr^{2+} ions dissolved from the glasses into Tris buffer solution as a percentage of the ions originally present in the glass. This indicates a glass solubility of less than 6.5% for all components at day 7. The solubility of 0Sr or 100Sr was higher than those of mixed CaO/SrO compositions, with the curves showing minima for the mixed compositions.

Figure 4(a) shows cell numbers on the glass powder compacts after culturing for 3 to 7 days. Increasing cell numbers were seen over time on both the control and glasses, showing increased cell proliferation. At days 3 and 5 of culture, cell numbers on the glasses were about twice as large as on the control; however, there were no significant differences with SrO substitution. After 5 days of culture, cell numbers on all samples were comparable, as the cultures had reached confluence. Cell doubling time (DT) between seeding and 3 days of culture were calculated

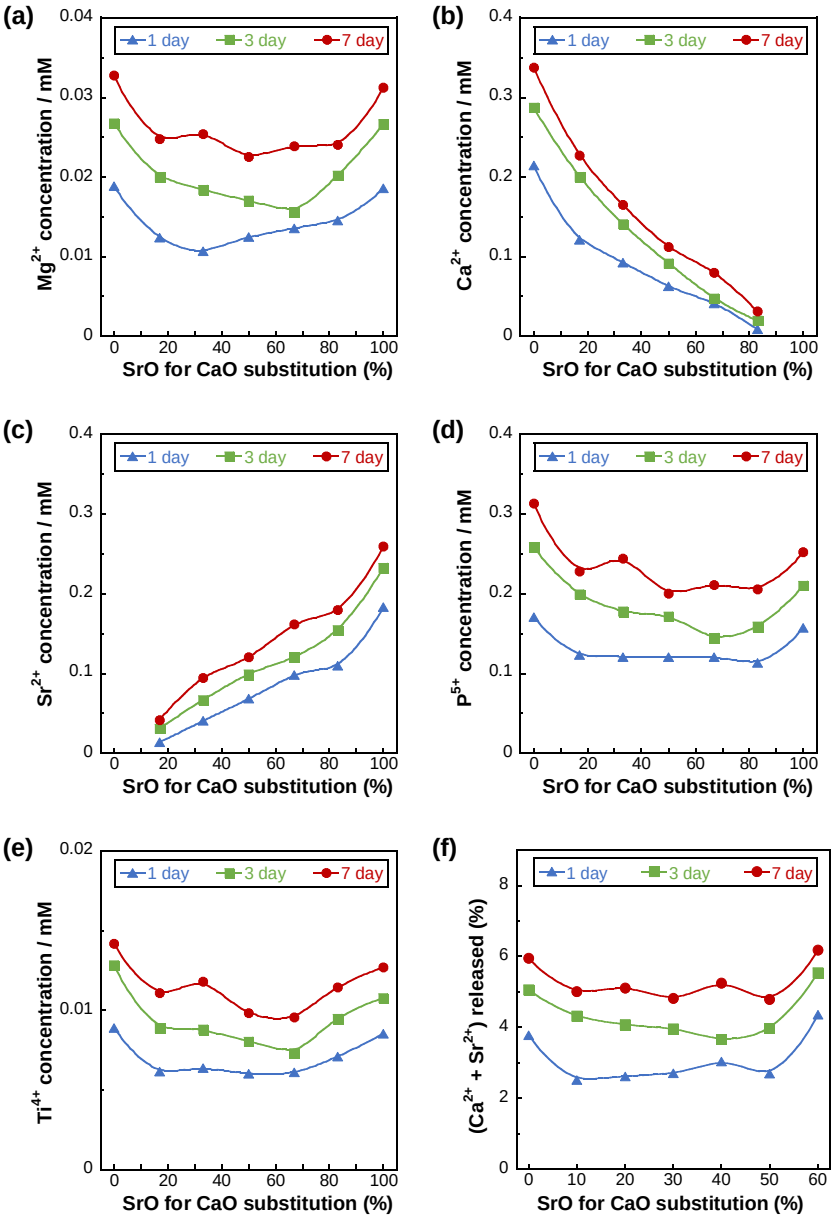

Figure 3: Released ions amount in Tris buffer solution from the *y*Sr glasses. The amounts of (a) Mg^{2+}, (b) Ca^{2+}, (c) Sr^{2+}, (d) P^{5+} and (e) Ti^{4+} ion, and (f) the dissolution rate (percentage) of the sum of Ca^{2+} and Sr^{2+} ions. The dissolution rate was calculated using the following equation, Dissolution rate $(\%) = \frac{1000\times\left(\frac{z}{W_{Atom}}\right)}{([Atom_{Rate}]\times W_{sample})/(M_{glass}\times V_{solution})} \times 100$, where z (ppm) is the ion amount dissolved in the Tris buffer solution after soaking, measured by ICP-AES. W_{Atom} is the atomic weight of the ion, and $[Atom_{Rate}]$ is the atomic rate of the ion calculated from the nominal glass composition. W_{sample} (g), M_{glass}, and $V_{solution}$ (L) are the weight of the sample soaked, the molecular weight of the glass, and the volume of Tris buffer solution, respectively.

with the following equation,

$$DT = (t - t_0) \cdot \log 2 / (\log N - \log N_0) \quad (1)$$

where, t is time (h), t_0 is initial time (h), N is number of cells at t and N_0 is number of cells at t_0. The DT of the control and the glasses were 24 and 16 hours, respectively (Fig. 4(b)). The glasses showed shorter DT compared with that of the control, but there were no significant differences between SrO-substituted samples. Fig. 4(c) shows ALP expression levels of cells cultured on the glass powder-compacts at days 7 and 10. At day 7 of culture, there were no significant differences in ALP expression between SrO-substituted samples. At day 10 of culture, ALP expression seemed to increase with low SrO substitution, but was comparable for the ≥ 50%-substituted glasses.

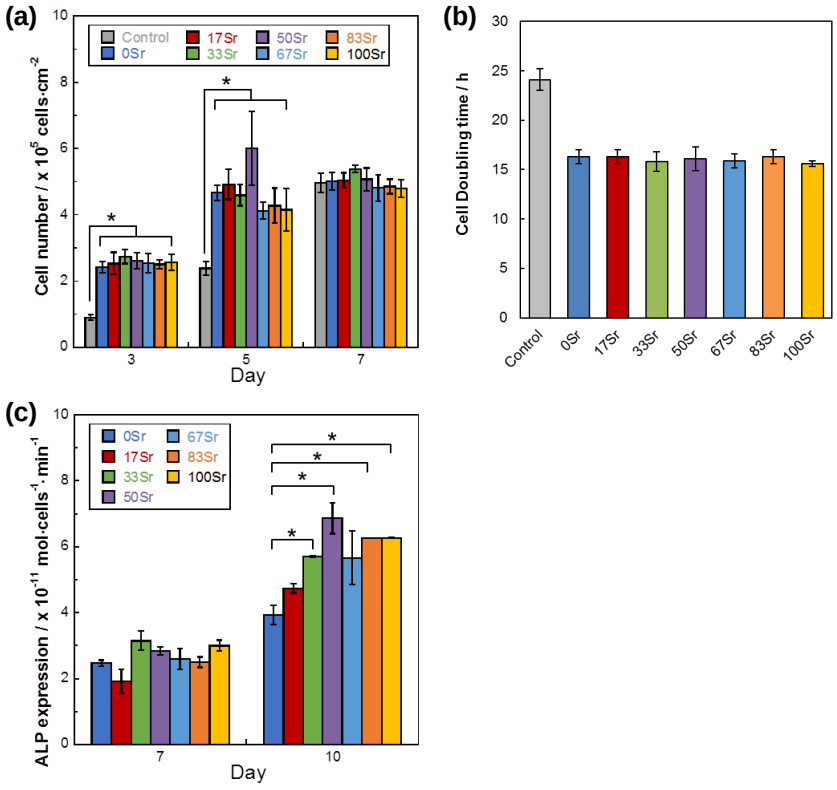

Figure 4: (a) Cell numbers after 3–7 days of culture on the *y*Sr glass powder compacts. (b) Cell doubling time between seeding and 3 days of culture on the *y*Sr powder compacts. (c) ALP expressions of the *y*Sr glass powder compacts after 7–10 days of culture. ($^*P < 0.05$, Student's t test)

4 Discussion

Phosphate glasses are known to dissolve in water, allowing for the release of therapeutically active ions, and they have been investigated for use as degradable implant materials and controlled release materials [23]. Their dissolution rate depends strongly on phosphate content and structure [24], with invert glasses, containing very low phosphate contents and mostly consisting of ortho- and pyrophosphate groups, showing the most controlled dissolution [25]. Unfortunately, invert glasses also have a very pronounced tendency to undergo crystallization upon heat treatment [16], making their sintering challenging.

In a previous work, we examined glass formation of invert glasses in the system $7Na_2O{\cdot}(60-z)CaO{\cdot}z$ $SrO{\cdot}30P_2O_5{\cdot}3TiO_2$ (mol%; z = 0 to 60 mol%). Our results showed that it was difficult to obtain glasses in an amorphous state, owing to spontaneous crystallization occurring during quenching. This was confirmed by DTA results, which showed that the glasses had low glassification degrees between 0.02 and 0.05 [14]. The glassification degree is a parameter describing the glass forming ability of a system, previously described by Ouchetto *et al.*, who showed that the glassification degree of zinc phosphate glasses in the metaphosphate compositional range had values between 0.20 and 0.25, while invert glasses had much lower values between 0.07 and 0.12 [20]. This, again, shows a more pronounced crystallization tendency (and thus lower glass forming ability) of invert glasses compared to metaphosphate ones. Our previous work also showed that the glass forming ability decreased with increasing SrO substitution [14]. This was most likely caused by the lower field strength (owing to a larger ionic radius) of Sr^{2+} compared to Ca^{2+}, which has been shown to affect various glass properties in silicate [26, 27] and phosphate glasses [28], owing to changes in packing density [26].

The glassification degrees of the glasses examined in the present work showed values between 0.05 and 0.08, which, while still being low compared to metaphosphate glasses, are almost twice as large as those in our previous work [14]. This was possible by replacing Na^+, a low field strength cation, with Mg^{2+}, resulting in much easier glass formation [19] and highly reduced spontaneous crystallization. According to the Dietzel's rule, MgO is an intermediate oxide [29], and it has been suggested that in bioactive

silicate glasses, some Mg^{2+} ions in the glass may switch their role from network modifier to network former [30]. Karakassides *et al.* suggested that Mg^{2+} ions act as network former in phosphate glasses with high MgO content, consisting of phosphate Q^0 and Q^1 groups [21]. Also, in our previous work, a magnesium phosphate invert glass showed better glass formation compared with a calcium phosphate or strontium phosphate one [17]. MgO substitution in phosphate invert glasses is a good method to improve glass formation, since Mg has a high field strength and may possibly enter the phosphate network to form P-O-Mg bonds, which crosslink orthophosphate groups (Q^0) and short phosphate chains (Q^1) [18] to form longer structures.

The field strength differences between Mg^{2+} and Na^+ (Na^+: 0.19, Mg^{2+}: 0.45 or 0.53 (4-fold or 6-fold coordination) valence/Å^2 [29]) caused the slight blue-shift of the phosphate bands in the Raman spectra of glass 0Sr compared with Ca-IG, which has also been observed when replacing Ca with Mg [18]. Besides that, there were no significant differences between the spectra of 0Sr and Ca-IG. With increasing SrO content in the glasses, the opposite effect was observed and the phosphate bands red-shifted, since the Sr^{2+} ion has smaller field strength than the Ca^{2+} ion (Ca^{2+} : 0.33, Sr^{2+} : 0.28 valance/Å^2) [29]; these shifts indicate a similar trend as observed previously [14].

In the present work, the incorporation of MgO (7 mol%) instead of Na_2O also resulted in much lower ion release from the glasses compared with our previous findings [14]. This is also caused by the higher field strength of Mg^{2+} ions and the higher packing density (owing to replacement of large Na^+ ions by small Mg^{2+} ions), which increased the chemical durability of the glasses [18]. All glass components in this work dissolved at similar rates; the glasses showed congruent dissolution, indicating no selective ion leaching from the glasses. The relatively low solubility of the glasses (up to 6% dissolution over one week) is caused by a combination of factors, including the low phosphate content [25], the absence of alkali ions and the presence of high field strength intermediate ions, such as Mg^{2+} and Ti^{4+} [31, 32], which increase glass durability.

Mixed CaO/SrO glasses in the present work showed minimal ion release curves for the mixed compositions. This effect is also observed for the trends of T_g and T_c, and has been previously observed for CaO- and SrO-containing phosphate glasses [14, 18]. This also resembles the trends observed for mixed alkali glasses [33, 34] and may indicate the presence of a mixed cation effect in the present glass system. The mixed alkali effect is still not fully understood, but attempts have been made to explain it by suggesting that alkali ion mobility is inhibited [35], owing to the ions blocking each other's pathway and thus inhibiting ion migration [36]. While the mixed alkali effect is well known, a mixed cation (or mixed alkaline earth) effect is much more questionable. For MgO-substituted glasses a similar trend has been observed for thermal properties and ion release [18], and herein changes in Mg coordination number with increasing MgO for CaO substitution may explain the effect, as Mg is known to occur in six- and four-fold coordination. However, changes in coordination number of Mg^{2+} ions in phosphate glasses have also been questioned [37, 38]. Thus, we cannot fully explain these observations at present.

Cell numbers on the glasses were twice larger than that of the control at day 3 and 5, owing to either faster cell proliferation or higher initial cell attachment on the glasses compared with the control. Cell doubling time was also 1.5-times faster on the glasses than the control. In contrast, there was no significant difference with glass composition, *i.e.* SrO for CaO substitution, in the present work. While these results show a very positive and promising effect of the glasses, they cannot currently be fully explained and require further investigation. There are, however, a number of possible explanations, which we will address in the following text. As the effect did not appear to vary much with glass composition, it is most likely that the release of Sr^{2+} ions is not the dominating factor affecting cell attachment or proliferation. Takeichi *et al.* reported that Mg^{2+} ions accelerate cell attachment [4], and according to Wolf *et al.*, Mg^{2+} ions enhance the proliferation of HL-60 cells [6]. Mg^{2+} ion release from the glasses may thus have enhanced cell adhesion and subsequent proliferation.

In contrast, ALP activity increased slightly in the order of 0Sr < 17Sr < 33Sr to 100Sr at day 10, so Sr^{2+} ions may have played a more pronounced role in ALP activity than in cell attachment and proliferation in the present work. Indeed, Barbara *et al.* reported that 0.1 to 1 mM of Sr^{2+} ions promotes ALP activity and collagen synthesis in MC3T3-E1 cells [39], and Sr^{2+} ions released from bioactive glasses have been shown to increase osteoblast metabolic activity and ALP activity [15]. Sr^{2+} ion release from the SrO-substituted glasses may thus play an important role in enhancing cell differentiation, while Mg^{2+} ions have also been reported to enhance cell differentiation [7] and may have had an effect in our experiments.

In summary, MgO-CaO-SrO-TiO_2-P_2O_5 invert glasses were successfully prepared and their dissolution behavior and cell response were evaluated. Through incorporation of MgO, their glass formation was improved compared to previous works, an effect that can be explained by the high field strength of Mg^{2+} ions, which stabilizes the glass network. Osteoblast-like cells (MC3T3-E1) showed larger cell

numbers attached on the glasses and increased DTs compared with the control, while ALP activity was enhanced for higher SrO content in the glass.

These positive effects may be associated with the release of Mg^{2+} and Sr^{2+} ions from the glasses, and will be investigated further in our future works.

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