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BIOCOMPATIBILITY STUDIES

The influence of SrO and CaO in silicate and phosphate bioactive glasses on human gingival fibroblasts

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Abstract In this paper, we investigate the effect of substituting SrO for CaO in silicate and phosphate bioactive glasses on the human gingival fibroblast activity. In both materials the presence of SrO led to the formation of a CaP layer with partial Sr substitution for Ca. The layer at the surface of the silicate glass consisted of HAP whereas at the phosphate glasses it was close to the DCPD composition. In silicate glasses, SrO gave a faster initial dissolution and a thinner reaction layer probably allowing for a continuous ion release into the solution. In phosphate glasses, SrO decreased the dissolution process and gave a more strongly bonded reaction layer. Overall, the SrO-containing silicate glass led to a slight enhancement in the activity of the gingival fibroblasts cells when compared to the SrO-free reference glass, S53P4. The cell activity decreased up to 3 days of culturing for all phosphate glasses containing SrO. Whereas culturing together with the SrO-free phosphate glass led to complete cell death at 7 days. The glasses containing SrO showed rapid cell proliferation and growth between 7 and 14 days, reaching similar activity than glass S53P4. The addition of SrO in both silicate and phosphate glasses was assumed beneficial for proliferation and growth of human gingival fibroblasts due to Sr incorporation in the reaction layer at the glass surface and released in the cell culture medium.

1 Introduction

The potential of bioactive glasses S53P4 (BonAlive®) as bone regeneration graft has been verified in several clinical studies [1, 2]. The reaction mechanisms of silicate bioactive glasses have been extensively studied and are nowadays well understood [3]. In addition, doping the glasses with other ions such as strontium, magnesium, copper and zinc are known to affect healing and growth of vascularized bone tissue [4].

Phosphate glasses have been found good alternatives to silicate glasses for example in bone repair and reconstruction [5]. Even more, it is well known that the composition of the phosphate glasses can be tailored to possess low melting temperatures, high concentrations of rare earths or other metal ions [6, 7].

Strontium ions have therapeutic effect on osseous healing and they are used as strontium ranelate for treating osteoporosis [8]. SrO addition in silicate glasses is beneficial in vitro and in vivo for the replication of pre-osteoblastic cells while decreasing the activity of osteoclasts [9]. Sr^{2+} ions have been found earlier to induce osteoblasts activity and thus stimulate bone formation and also to reduce bone resorption [10, 11]. The presence of SrO in sol–gel derived silicate bioactive glasses was investigated recently [12]. It was found that 5 wt% of SrO resulted in a significant up-regulation of Runx2, Osterix, Dlx5, collagen I, ALP, bone sialoprotein and ostocalcin mRNA levels associated with an increase of ALP activity and osteocalcin secretion [12]. In soda-lime glasses, SrO is a glass network modifier similar to

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CaO. In silicate glasses, substitution of SrO for CaO did not change the general glass structure expressed as network forming and modifying units [13]. However, the initial dissolution rate increased with SrO content in the glass and lead to slightly higher pH values of the immersion solution simulated body fluid. Glass with 5 mol% SrO formed similar thick and distinct silica-rich and CaP layers at the surface of the SrO-free reference glass S53P4. However, the higher the strontium content in the glass, the thinner and the more blurred layers formed at the glass surface. The results suggested that substituting SrO for CaO leads to thin Sr-substituted apatite layer at the glass surface [13]. In phosphate glasses, substituting SrO for CaO led to significant changes in the thermal properties. When immersed in SBF, an apatite-like layer with increasing thickness as a function of the immersion time formed on all phosphate glasses, independently of the glass composition. However, the concentration of phosphate ions released into the solution during the immersion was less for the SrO-containing glasses. Accordingly, the pH of the immersion solutions for the SrO containing glasses changed less. The concentrations of SrO and MgO in the reaction layer at the glass surfaces increased with the SrO content in the glass. The presence of SrO and MgO in the apatite layer was assumed to limit phosphate leaching in the solution and thus to increase the durability of the phosphate glasses [14].

This study aims to evaluate the effect of replacing CaO with SrO in both silicate and phosphate bioactive glasses on the proliferation and activity of human gingival fibroblasts. First, the glasses were immersed in the culture medium without cells to study the reaction mechanisms compared to their reactions in the simulated body fluid. Then, cells were introduced in the medium and their proliferation and activity was analyzed using the protocol described in [15]. The cell activity and proliferation in the silicate and phosphate glasses were correlated to their SrO content and to the changes in the dissolution speed in the culture medium.

2 Experimental procedure

2.1 Glass processing

The glasses were melted in platinum crucibles. The phosphate glasses were prepared using the analytical grade reagents $NaPO_3$, $SrCO_3$ $CaCO_3$ and $(NH_4)_2HPO_4$, as reported in [14]. The nominal composition of the glasses is (mol%) $50P_2O_5$–10 Na_2O–(40 − x)CaO–xSrO with x varying from 0 to 40. The glasses were coded according to the SrO content (PSr0, PSr20, PSr26.67 and PSr40).

The silicate glasses were melted from batches mixed of sand (99.4 % pure SiO_2), and analytical grades of Na_2CO_3, $Sr(NO_3)_2$, $CaCO_3$, and $(NH_4)H_2PO_4$. The nominal oxide composition of the experimental glasses is (mol%) $53.85SiO_2$–$22.66Na_2O$–$1.72P_2O_5$–(21.77 − x)CaO–xSrO with x varying from 0 to 10. The glasses were coded according to the SrO content: SSr0 = S53P4, SSr5 and SSr10. The melting procedure has been described elsewhere [13].

All glasses were quenched in a pre-heated graphite mold in order to obtain a cylinder/rod of the length 10 cm and diameter 1 cm. After casting, the glasses were annealed at 40 °C below their respective glass transition temperature for 12 h. The annealing temperature and time were chosen to allow removal of internal stresses due to quenching while minimizing the risk for surface nucleation. Samples were cut and polished to a thickness of 2 mm. The oxide composition of the glasses measured via SEM/EDX agreed with the nominal composition within the accuracy of the measurements.

2.2 Cell proliferation

Human gingival fibroblasts [16] were maintained in DMEM supplemented with 10 % fetal bovine serum and 100 U/μg penicillin–streptomycin (Gibco BRL, Life Technologies, Paisley, UK) and incubated at 37 °C in a 5 % CO_2 environment. Semi–confluent cultures were trypsinized, and the cells were counted and resuspended in complete culture medium. Culture medium (3 ml per well for the first 3 days and then 1 ml per well) was changed two to three times a week and the pH was measured before each medium change. Proliferation of cultured cells was determined using AlamarBlue™ assay (BioSource International, Camarillo, CA, USA) in colorimetric format. Fibroblasts were plated at a density of 25,000 cells/cm^2 on the substrates and cultured for up to 14 days. The substrates (n = 4) were withdrawn from the culture at predetermined times, and placed into sterile culture plates containing fresh culture medium with 10 % assay reagent. After 3 h of incubation, the absorbance values were read at 570 and 595 nm using an ELISA plate reader. Measured absorbances were used to calculate the reduction of assay reagent, and the cell proliferation rate were normalized in respect to the proliferation rate on the substrate of SSr0 glass (S53P4) at the first time–point, which was arbitrarily set to 100 %. The substrates were rinsed thoroughly three times with PBS and fixed for 1 h with 2.5 % glutaraldehyde. Then, all substrates were rinsed in buffer and dried in increasing alcohol series.

Three glass substrates per compositions were immersed for up to 14 days in the culture medium (without cells) using a similar experimental protocol than the one used in the cell culture test. The change in mass was measured and the composition of the formed reactive layer quantified.

The surfaces of the disks used in the cell culture medium with and without cells were analyzed using SEM/EDXA (Leo 1530 Gemini from Zeiss and EDXA from Vantage by Thermo Electron Corporation).

3 Results

The influence of the cell culture medium containing no cells on the reactions of the glasses was studied for up to 14 days. The weight loss of the samples as a function of immersion time is reported in Fig. 1a (silicate glasses) and b (phosphate glasses). All samples lost weight with increasing immersion time. For the silicate glasses no significant mass changes were measured for up to 3 days (Fig. 1a). However, with increasing immersion time the mass loss became significant. In addition, the mass loss slightly increased with increasing SrO content in the glass. In contrast, the mass loss of the phosphate glasses was significant already after one immersion day. For immersion times longer than 7 days, the SrO-free phosphate glass (PSr0) exhibited clearly higher mass loss than the SrO-containing glasses. While increasing the mass loss in silicate glasses, an addition of 20 mol% of SrO (PSr20) in phosphate glasses led to the smallest mass loss. However, at higher SrO contents, the mass loss of phosphate glasses slightly increased.

Figure 2 presents the SEM images of the surfaces of the silicate glasses SSr0 (a–c), SSr5 (d–f) and SSr10 (g–i) after 1, 7, and 14 days in the cell culture medium containing no cells. At 1 day of immersion, crystals appearing white in the image are present only at the surface of SSr0 (S53P4), whereas grains and areas with significantly different contrast can be seen at glasses SSr5 and SSr10. With increasing immersion time, significant changes in the surface contrast shown as bright and dark areas can be seen for all these glasses. SSr5 and SSr10 also exhibited bright spherical spots at immersion times longer than 7 days (not shown here).

SEM images of the surfaces of the phosphate glasses PSr0 (a, b and c), PSr20 (d, e and f), PSr26.67 (g, h and i) and PSr40 (j, k and l) at 1, 7 and 14 days in the cell culture medium containing no cells are shown in Fig. 3. At 1 day, a distinct layer is seen at the surface of all glasses. This layer seems to be rather thin, brittle and non-adherent at the surface of all glasses except PSr26.67. At longer times than 3 days the samples were covered by rather uniform layers.

Cell proliferation tests were done using culture medium containing human gingival fibroblasts. The pH of the medium was measured at 1, 3, 5, 7, 10 and 14 days. Figure 4 presents the pH of the medium on the silicate glass substrate (a) and the phosphate glass substrate (b) as a function of cell culture time. The pH was measured before changing the medium. Figure 4a exhibits an increase in the pH at day one compared to the initial pH (pH = 7.3) of the fresh culture medium. After the successive changes of the culture medium in wells, the increase in the pH of the medium reduced and seemed to level off close to the physiological pH (~7.4) (Fig. 4). For the silicate glasses, lower decrease in the pH was measured with increasing strontium oxide content. After the first increase in the pH from the value of the initial culture medium, the pH of the medium decreased upon immersion of the phosphate glasses (Fig. 4b). At each time point, the pH values seemed to depend only slightly on the SrO content in the glass. The pH of the cell culture medium decreased with increasing immersion time also for the phosphate glasses. However, the decrease did not correlate with the SrO content in the glass.

The surface of the samples immersed in the culture medium containing human gingival fibroblasts was

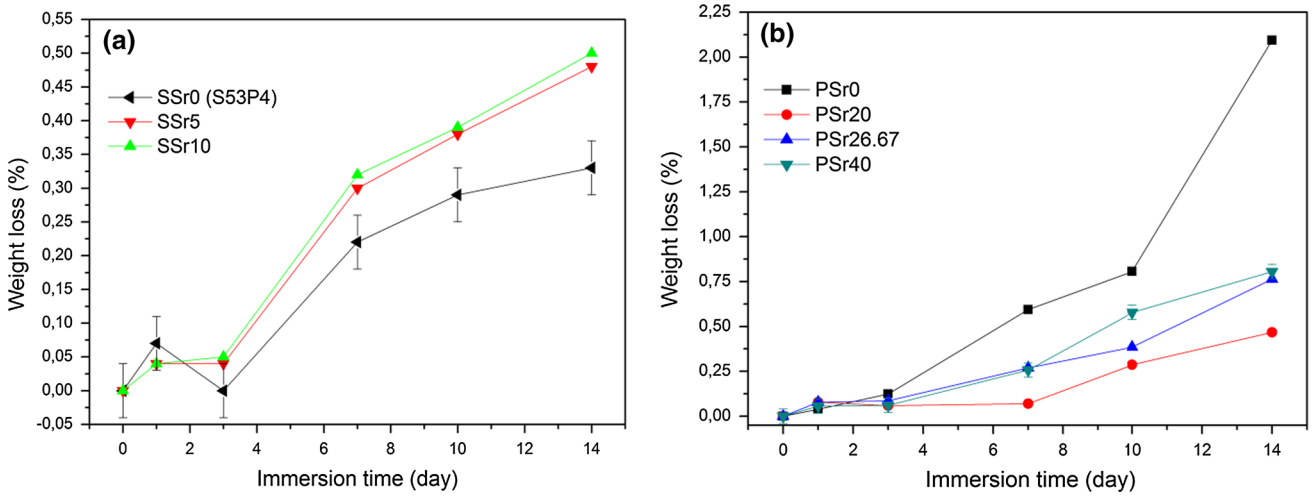

Fig. 1 Weight loss (%) of silica **a** and phosphate **b** bioactive glasses as a function of immersion time in culture media

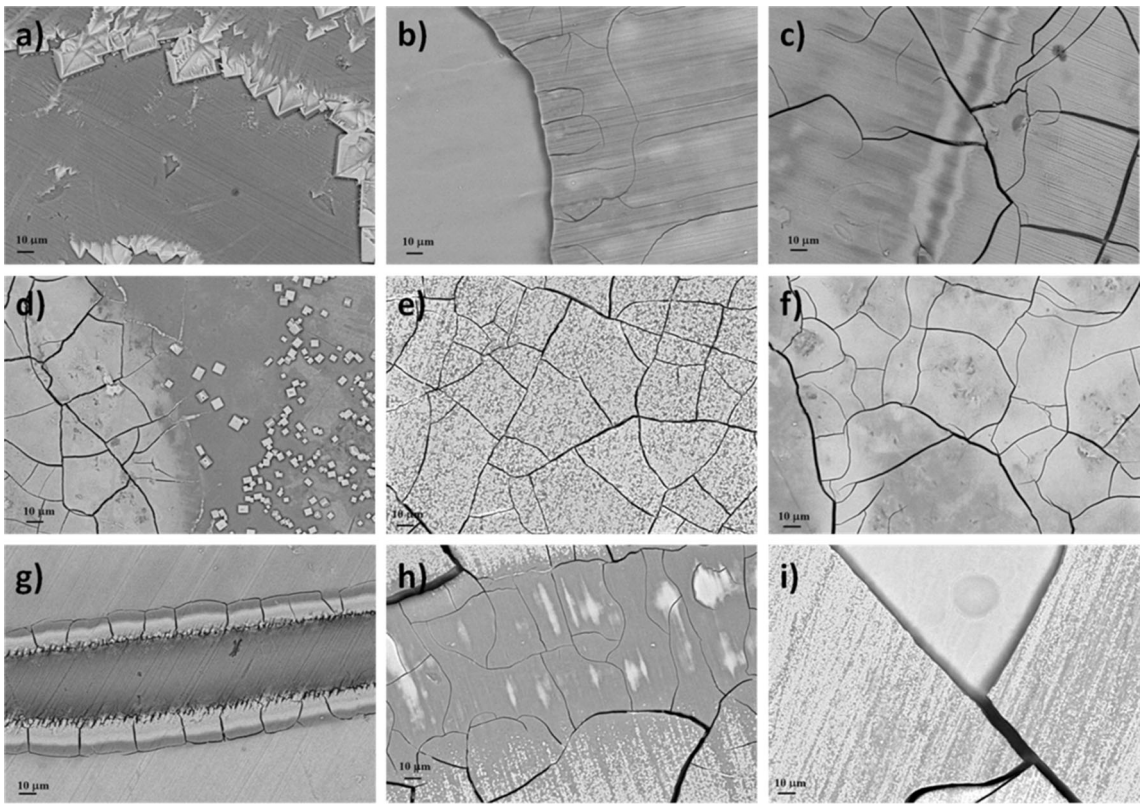

Fig. 2 SEM images of the surface of silicate glasses after immersion in cell culture medium for 1, 7 and 14 days: SSrO (**a**, **b**, **c**), SSr5 (**d**, **e**, **f**), SSr10 (**g**, **h**, **i**)

analyzed using SEM. Figure 5 presents the SEM images of the silicate glasses SSr0 (S53P4) (a–c), SSr5 (d–f) and SSr10 (g–i) at 1, 7 and 14 days of cell culture. After 1 day, flattened and elongated cells could be seen at the surfaces of all glasses. The number of cells increased with time and from day 7 the cell coverage was uniform over the surface.

Figure 6 presents the SEM images of the phosphate glasses PSr0 (a–c), PSr20 (d–f), PSr26.67 (g–i) and PSr40 (j–l) at 1, 7 and 14 days of immersion with the cells. After one and 7 days, a few rounded cells could be seen only at the surface of the glass PSr0. After 7 days elongated cells could be seen at the surface of all but glass PSr0. At the time points 10 and 14 days all samples but glass PSrO were fully covered by cells.

The proliferation and activity of the gingival fibroblast are given as normalized cell activity at the surface of the silicate and phosphate glasses in Fig. 7. The cell activity was set to 100 % for the glass SSr0 (S53P4) at 1 day of immersion. All samples are then compared to this value. The cell activity increased for all silicate glasses with culture time. After 7 days, the activity of the cells at the SrO-containing glasses was higher than at SSr0 (S53P4) ($P < 0.05$ using one-way ANOVA and Tukey mean comparison). In contrast, some cell activity was detected at the phosphate glass PSr0 only after 1 day. However, the phosphate glasses containing SrO showed first a decrease in the cell activity for the first 3 days after which an increase for longer immersion times was measured.

4 Discussion

4.1 Silicate glasses

The mass of the silicate glasses decreased almost linearly with increasing immersion time in the cell culture medium (Fig. 1a). This indicates that the glasses dissolve in the cell culture medium similarly as in other buffered aqueous solutions [13, 17]. In addition, the glasses containing SrO show higher weight loss than the SrO-free composition SSr0 (S53P4). This result can be explained with a faster initial dissolution rate due to expansion of the glass network structure induced by the substitution of SrO for CaO [13]. SEM-EDX analysis of the glasses showed some NaCl crystals at the surface of SSr0 (S53P4) and SSr5 after 1 day in the cell culture medium (Fig. 2). The formation of NaCl crystals in DMEM has been recently discussed in [18]. EDX analyses of the glass surfaces was performed and

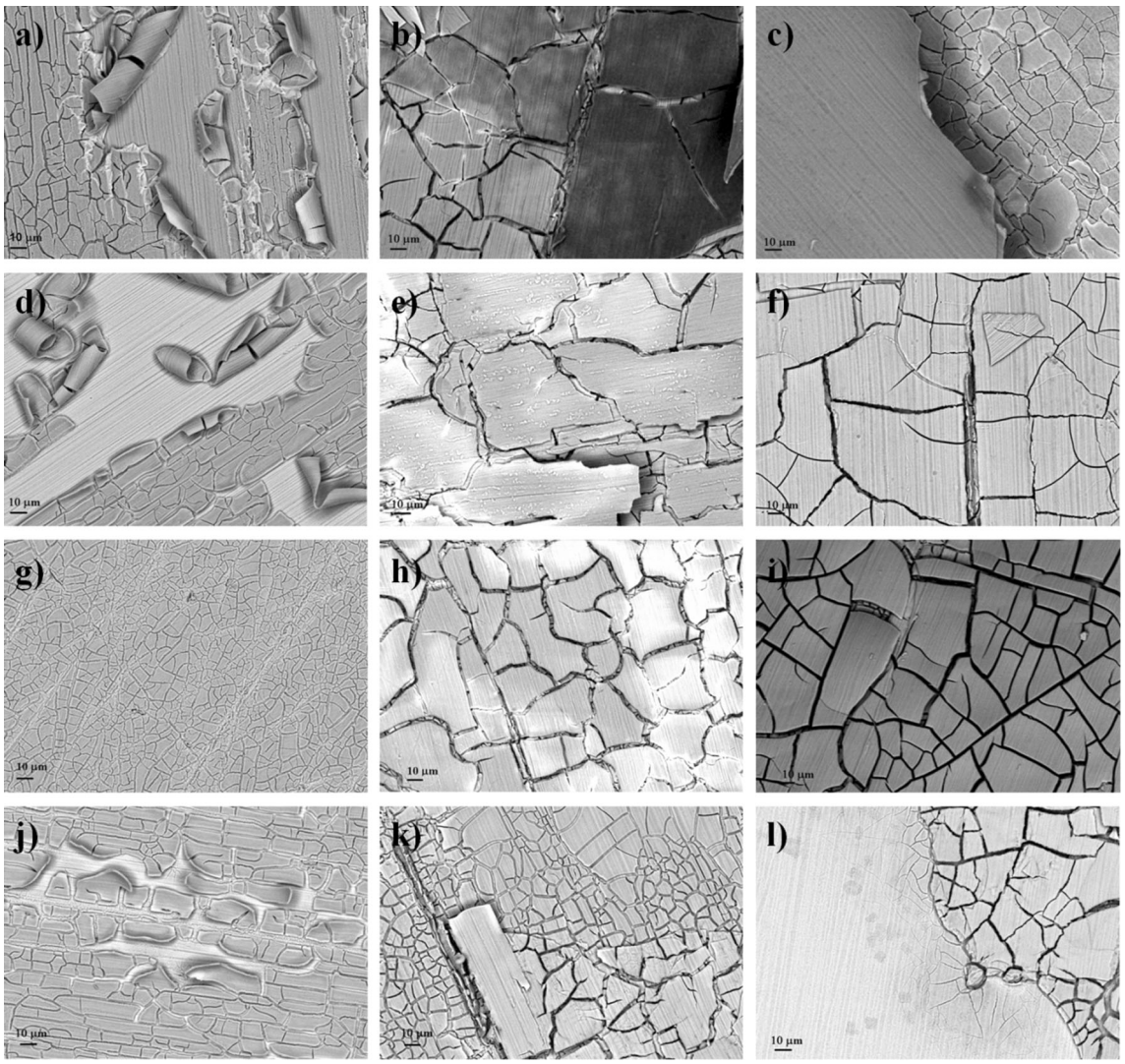

Fig. 3 SEM images of the surface of phosphate glasses after immersion in cell culture medium for 1, 7 and 14 days: PSrO (**a**, **b**, **c**), PSr20 (**d**, **e**, **f**), PSr26.67 (**g**, **h**, **i**), PSr40 (**j**, **k**, **l**)

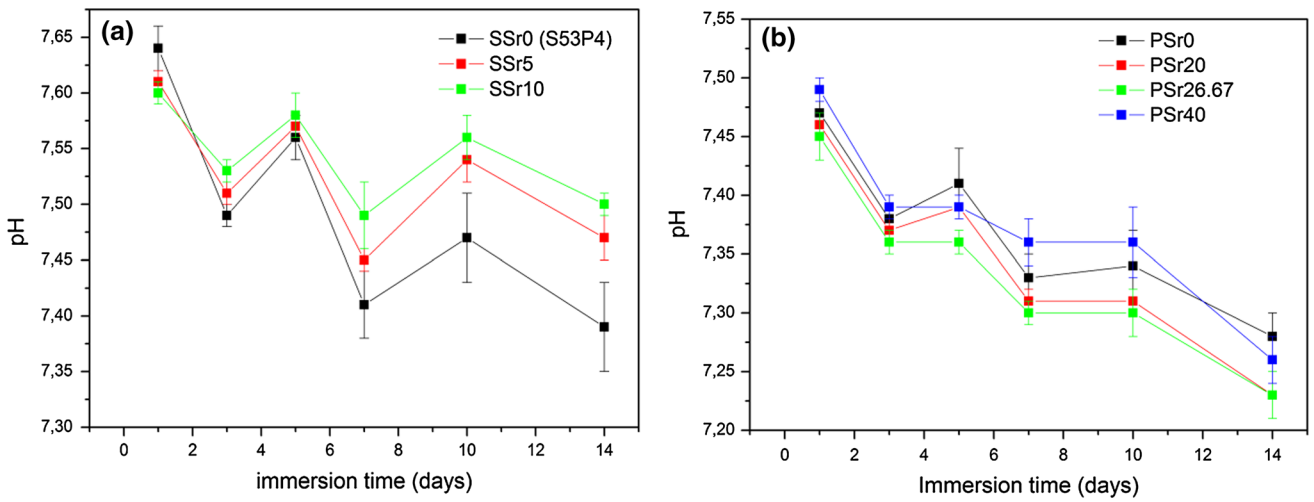

Fig. 4 pH of the culture medium as a function of immersion time **a** silicate glasses, **b** phosphate glasses

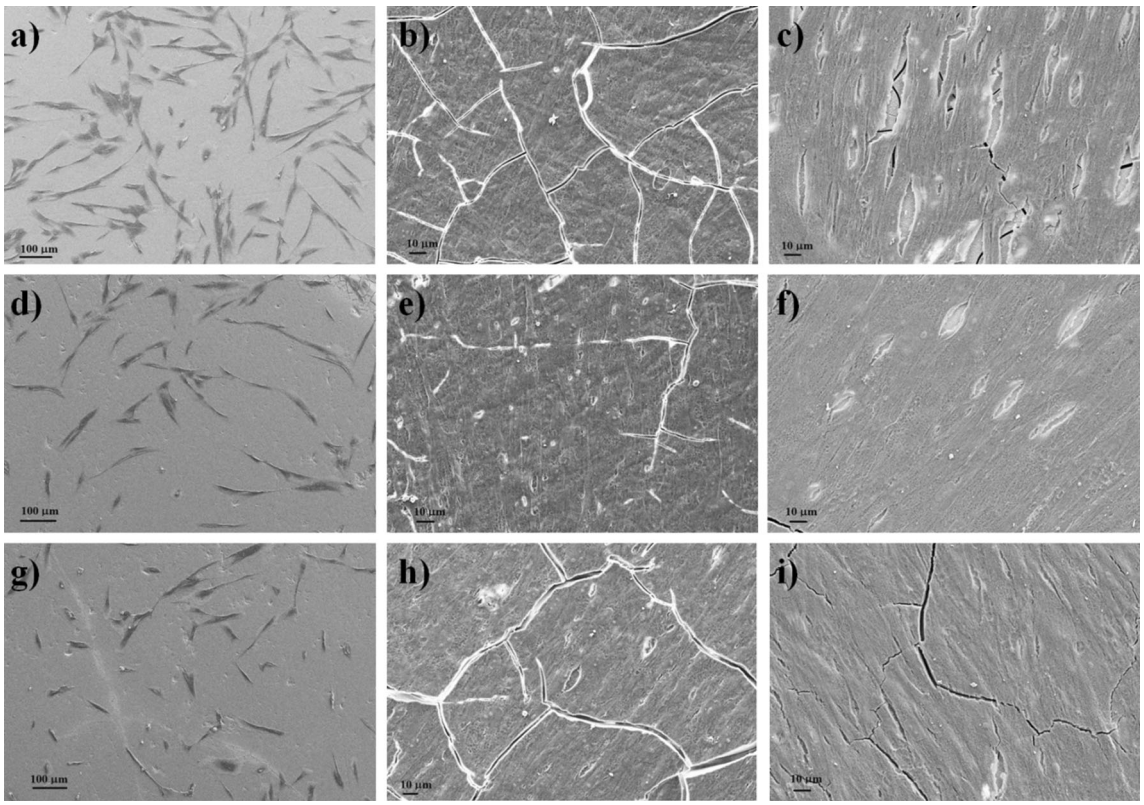

Fig. 5 SEM images of the surface of silicate glasses after cell culture for 1, 7 and 14 days: SSrO (**a**, **b**, **c**), SSr5 (**d**, **e**, **f**), SSr10 (**g**, **h**, **i**)

revealed a decrease of the Si, Ca and Na content, whereas the amount of P ions increases with increasing immersion time. This behavior was independent of the glass composition. Such changes can be attributed to the precipitation of calcium phosphate layer at the surface of the glasses (brighter areas in the SEM images) with some areas rich in SiO_2 (darker areas in the SEM images). The formed surface layer contained also $\sim$2 mol % of MgO. The MgO oxide is believed to come from the culture medium and it partially replaces Ca in the calcium phosphate layer. 3 mol% (SSr5) and 6 mol% (SSr10) of SrO were also present in the layer. The presence of SrO was expected based on our previous results [13]. Based on the composition analysis the (Ca + Sr + Mg)/P ratio was calculated and is presented in Fig. 8a as a function of immersion time. The ratio decreases with immersion time and levelled off to a value of around 1.6, similarly as observed when immersing the glass in SBF or TRIS-buffer [13]. Thus, in the cell culture medium, hydroxyapatite formed at the surface of the bioactive silicate glasses independently of the SrO content.

The glasses were incubated also in the cell culture medium containing human gingival fibroblasts. The pH was measured regularly prior to partial change of the medium. At day one the pH of the media containing the bioactive silicate glasses increased to 7.6–7.65. Over time the increase in the pH of the medium decreased. At day 14 almost no change in the pH values prior to and 2 days after changing the medium was evidenced. Such a slowing down of the dissolution reactions is believed to take place as the reaction layer forms at the particle surfaces and acts as a barrier to the dissolution. Interestingly, the higher the SrO content in the glass, the faster cell growth (Fig. 5a, d, and g) and the greater normalized cell activity compared to glass S53P4 was observed at the incubation time 7 days (Fig. 7). The highest changes in the pH values of the cell culture medium and the mass of the glass samples were observed between the time points 1 and 3 days both indicating glass dissolution. In addition, largest change in cell activity was observed between the time points 3 and 7 days, especially for the SrO substituted glasses. These observations suggest that the strontium ions released into the solution and present in the Sr-substituted HAP layer at the glass surface enhance cell adhesion and proliferation.

4.2 Phosphate bioactive glasses

Similarly to the silicate glasses, also the phosphate glasses exhibited a mass loss upon immersion in the cell-free culture medium (Fig. 1b). However, glass PSr0 showed a clearly higher wt% loss than the SrO containing glasses. As

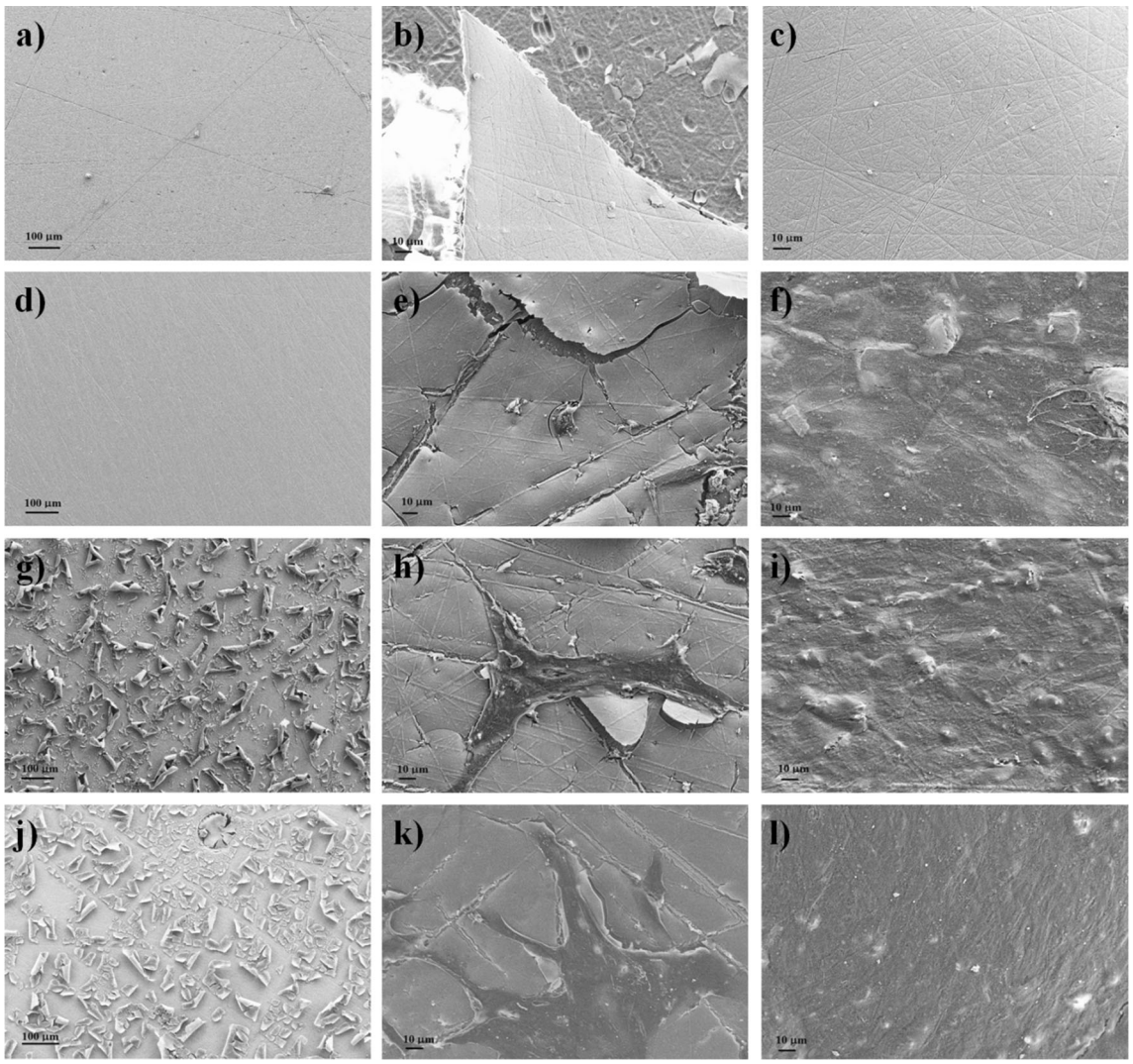

Fig. 6 SEM images of the surface of phosphate glasses after cell culture for 1, 7 and 14 days: PSrO (**a**, **b**, **c**), PSr20 (**d**, **e**, **f**), PSr26.67 (**g**, **h**, **i**), PSr40 (**j**, **k**, **l**)

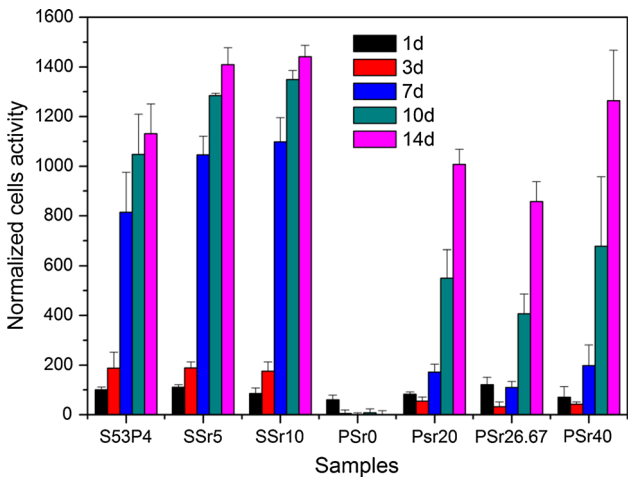

Fig. 7 Normalized cell activity at the surface of the bioactive silicate **a** and phosphate, **b** glasses

reported earlier, release of P and Ca in SBF was higher from PSrO than the SrO containing glasses [14]. Interestingly, the mass loss first decreased for the glass PSr20 and then increased with further increase of SrO content in the glass. This trend is in agreement with our previous study, which demonstrated that PSr20 reacted slowly in vitro [14]. The glass surfaces after various immersion times in the cell culture medium were analyzed by SEM (Fig. 3). According to SEM/EDXA, the P and Na content decrease whereas the Ca content increases compared to the nominal glass composition. The reaction layer at the glass surface consisted mainly of calcium, magnesium and phosphate for the strontium free composition, while the content of strontium in the surface increased with the strontium content in the glass. Interestingly, the layer was not properly attached to the strontium free composition, while strontium in the glass provided a better layer attachment. It is noteworthy that,

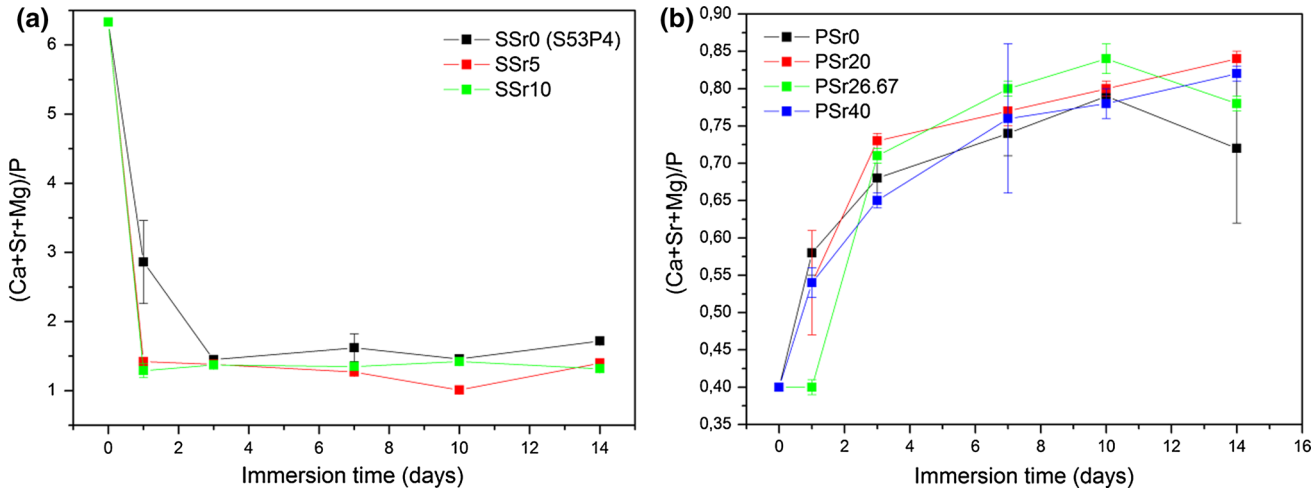

Fig. 8 (Ca + Sr + Mg)/P molar ratio at the surface of silicate **a** and phosphate **b** glasses as a function immersion time

while the MgO uptake at the surface of the silicate glasses was independent of the glass composition, the uptake of MgO in phosphate glasses increased with the SrO content in the glass. Furthermore, the more SrO in the glass, the more SrO was substituted for CaO in the layer, as also reported earlier [14]. The calculated (Ca + Sr + Mg)/P ratio as a function of immersion time is reported in Fig. 8b. The ratio increases from 0.4 to 0.8 independently of the glass composition. This is in agreement with our earlier results obtained when immersing the same glasses in SBF [14]. Thus, in the cell culture medium a Mg- and Sr-substituted CaP layer formed at the phosphate glasses. The layer was brittle at all compositions but better attachment was observed at the SrO containing glasses. The better chemical durability of the strontium containing glasses as suggested by the lower mass loss suggests that the slower dissolving glasses give a more stable substrate, i.e. better attachment for the alkali phosphate layer than the strontium free composition PSr0. This also suggests that SrO can be used to slow down the dissolution rate of phosphate glasses.

In the cell proliferation study of the phosphate glasses the pH of the culture medium decreased only slightly during the first 5 days (Fig. 4b). The rather low changes in the pH value over the whole test period was attributed to the phosphate release in solution and the simultaneous consumption of Ca and Mg ions for the precipitation of the CaP layer, thus preventing any large pH variations. The partly replenishing of the solution also explains the minor changes in the pH, this being also important for the cell viability. All glasses exhibited a similar change in pH, with immersion time, independently of the SrO content in the glass. The proliferation and morphology of the cells was studied by SEM/EDXA (Fig. 6). After 1 day of immersion in the cell culture media containing human gingival fibroblasts the reaction layer of the glasses seems to have partly detached from the surfaces (Fig. 6). Only a few cells could be seen at the surface of the glass PSr0. The cells appeared rounded and apoptotic after 1 day of cell culturing. With increasing time no cells could be found at glass PSr0 whereas at day 7 elongated cells were seen at the glasses containing SrO. The cells appeared to grow and proliferate and to cover the glass surface almost totally at the longest culture times (Fig. 6f, i, and l). The normalized cell activity at different culture time points (Fig. 7a) shows that the cell activity first decreased up to 3 days indicating an early death of the cells upon initial phosphate glass dissolution. However, after 7 days the cell activity appeared to increase and the growth and proliferation of the cells was fast. After 14 days, glasses PSr20 and PSr26.67 exhibited slightly lower cell activity than S53P4, whereas glass with PSr40 exhibited similar values as glass S53P4. Interestingly, no cell activity was observed at the surface of one glass (PSr0, 7 days). Skelton et al. have suggested three main factors which can explain the proliferation and growth of cells at the surface of the bioactive phosphate glasses [19]. They attributed the cell death to: (i) the glass dissolution process, (ii) the change in osmolarity of the medium, and/or (iii) the change in the pH of the culture medium. Indeed, the dissolution of bioactive phosphate glasses gives first leaching of Na^+ followed by hydration and hydrolysis of the phosphate chains [19, 20]. In our case, it appears that the pH change was independent of the SrO content. The experiments indicated differences in the mass loss depending on the glass composition. It was assumed that the rapidly dissolving phosphate glasses do not provide a proper substrate for cell adhesion and proliferation. In addition, the dissolving glasses give also differences in the osmotic concentration. Thus, too rapid dissolution of the glasses may lead to early cell death.

5 Conclusion

Substituting SrO for CaO in bioactive silicate and phosphate glasses significant changed their dissolution in vitro. In silicate glasses SrO tends to expand the glass network leading to an increased initial glass dissolution in SBF and in culture medium. Although formation of hydroxyapatite layer at the SrO containing glasses starts earlier than at the SrO free silicate glasses, the thickness of the layer was less after 2 weeks. Even more, the SrO containing glasses formed in SBF Mg- and Sr-substituted hydroxyapatite. AlamarBlue™ assay and SEM/EDXA of the glass surfaces suggested that SrO in the bioactive silicate glasses enhanced the activity of human gingival cells. The reasons for this enhanced cells growth and proliferation may be attributed to a more sustain glass dissolution over time, due to the thinner layer formation allowing for prolonged glass dissolution. In addition, Sr in the solution and incorporated into the hydroxyapatite layer may have a beneficial effect on human gingival cells.

In contrast, substitution of SrO for CaO in bioactive phosphate glasses lead to a slightly more cross linked glass network and thus slightly higher resistance to dissolution. The mass loss for the SrO-free glass was much greater than for the SrO-containing phosphate glasses. The fast dissolution of the SrO-free glass did not provide a proper substrate for the calcium phosphate surface layer to attach. Accordingly, the human gingival cells did not attach to and proliferate at the SrO free phosphate glass. This poorly bonded CaP layer along with large P release led to early cells death. However, the SrO containing phosphate glasses formed a surface layer better attached to the surface after a few days of immersion in the cell culture medium. This layer facilitated proliferation and growth of the human gingival cells. The cell activity increased after an initial decrease and was after 14 days of the same level of the same level for the SrO containing phosphate glasses and the SrO free silicate glass.

This study support that the presence of SrO in glass and in the reactive layer, as well as presence of Sr ions in the culture medium solution leads to enhanced gingival fibroblasts growth and proliferation both in silicate and phosphate bioactive glasses.

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