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Do bioactive glasses convey a disinfecting mechanism beyond a mere increase in pH — Source link

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Abstract

AIM: To test whether bioactive glasses kill microbiota via mineralization or the release of ions other than sodium. METHODOLOGY: Flame-spray synthesis was applied to produce nanometric glasses of different sodium content and constant Ca/P ratio: 28S5, 45S5 and 77S. Calcium hydroxide and nanometric tricalcium phosphate (TCP) were used as controls. Apatite induction was monitored by Raman spectroscopy. Bovine dentine disks with adherent Enterococcus faecalis cells were exposed to test and control suspensions or buffered solutions for 1 h, 1 day and 1 week. Colony-forming units were counted and disks were inspected using scanning electron microscopy. Suspension supernatants and solutions were analysed for their pH, osmolarity, calcium and silicon content. RESULTS: Sodium containing glasses induced pH levels above 12, compared with less than pH 9 with sodium-free 77S. Calcium hydroxide, 45S5 and 28S5 killed all bacteria after 1 day and lysed them after 1 week. TCP caused the highest apatite induction and substantial calcification on bacteria adhering to dentine, but did not reduce viable counts. 77S achieved disinfection after 1 week without visible apatite formation, whilst the buffer solution at pH 9 caused only minimal reduction in counts. CONCLUSION: Bioactive glasses have a directly and an indirectly pH-related antibacterial effect. The effect not directly linked to pH is because of ion release rather than mineralization.

Do bioactive glasses convey a disinfecting mechanism beyond a mere increase in pH?

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Key words: Disinfection, bioactive glass, nanoparticles, *Enterococci*

Running title: Bioactive glass as disinfectant

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Abstract

Aim Bioactive glasses of the SiO_2-Na_2O-CaO-P_2O_5 system appear to exert antimicrobial effects by creating an alkaline environment mainly via their release of sodium ions. However, non pH-related effects on infected dentine have been suspected. This study tested whether bioactive glasses also kill microbiota via mineralization or the release of ions other than sodium.

Methodology Flame-spray synthesis was applied to produce nanometric glasses of different sodium content and constant Ca/P ratio: 28S5, 45S5, and 77S. Calcium hydroxide and nanometric tricalcium phosphate (TCP) were used as controls. Apatite mineral induction was monitored by Raman spectroscopy. Standardized bovine dentine disks with adherent *Enterococcus faecalis* cells were exposed to test and control suspensions or buffered solutions for 1 hour, 1 day, and 1 week. Colony-forming units were counted, and disks were inspected using scanning electron microscopy. Suspension supernatants and solutions were analyzed for their pH, osmolarity, calcium and silica content.

Results Sodium containing glasses induced pH levels above 12, compared to less than pH 9 with the sodium-free 77S. Calcium hydroxide, 45S5, and 28S5 killed all bacteria after 1 d, and lysed them after 1 w. TCP caused the highest apatite induction and substantial calcification on bacteria adhering to dentine, but did not reduce viable counts. 77S achieved disinfection after 1 w without visible apatite formation, whilst the buffer solution at pH 9 caused only a minimal reduction in counts.

Conclusion Bioactive glasses have both a not and a directly pH-related antibacterial effect. The former is due to ion release rather than mineralization.

Introduction

Bioactive glasses are potentially interesting materials in dentistry not only because of their ability to mineralize dentine (Forsback *et al.* 2004; Vollenweider *et al.* 2007) but also due to their antimicrobial effect in closed systems (Sepulveda et al. 2002; Stoor *et al.* 1998). Consequently, bioactive glasses could potentially be used as topical endodontic disinfectants without the purported negative side effects of calcium hydroxide on dentine stability (Doyon *et al.* 2005). The short-term antimicrobial effect of these glasses has been attributed exclusively to their ability to raise pH in an aqueous environment (Allan *et al.* 2001). This pH increase results from the release of alkali ions, mainly Na^+, and the incorporation of protons (H^+) into the corroding material (Ratner 2004; Sepulveda *et al.* 2002). Therefore a glass containing more sodium is able to generate a higher pH in solution (Wallace *et al.* 1999).

As recently shown, preparation of ultrafine bioactive glasses by a dry synthesis pathway called flame spray synthesis is possible and allows the preparation of different bioactive glasses with varying composition and in nanoparticulate form (Brunner *et al.* 2006). The resulting nanoparticulate glass with the classical 45S5 composition (Bioglass®) showed a higher dissolution rate of alkaline species and thus an elevated antimicrobial efficacy *in vitro* when compared to the currently available melt-derived, micron-sized bioactive glass whilst having the same chemical composition (Waltimo *et al.* 2007). However, studies with infected dentine blocks have suggested that there might be other, not directly pH-related, effects with a slower onset promoted by bioactive glass particles in a liquid environment that affect bacterial viability (Zehnder *et al.* 2004). Recent observations suggested that these effects might either be related to an

encapsulation of the bacteria adhered to dentine by mineralization (Zehnder *et al.* 2006a) or the sustained release of ionic species from the glass during corrosion (Zehnder *et al.* 2006b).

Using nanoparticulate bioactive glasses with varying sodium and silicon content and a constant Ca/P ratio, we tested the following hypotheses: i) the immediate killing effect of glasses on microbiota is related to their sodium content and thus their alkaline capacity; ii) the effect with a slow onset after several days is related to apatite precipitation on the bacteria; or iii) the latter effect is promoted by soluble ionic species rather than the calcium and phosphate ions mentioned in hypothesis ii).

Materials and methods

Preparation of test and reference materials

Nanometric bioactive glasses with the compositions 28S5, 45S5, and 77S (Table 1) were prepared from suitable precursor solutions (Grass *et al.* 2005; Stark *et al.* 2003) as described in more detail by Brunner and coworkers (Brunner *et al.* 2006). In these compositions the sodium content is variable (from high to no sodium) and a constant Ca/P ratio is preserved.

Nanoparticulate tricalcium phosphate (TCP) was produced according to Loher and coworkers (Loher *et al.* 2005) as a reference material, to assess the impact of a nanometric substance with minimal change in pH but high mineral induction on bacterial viability. The shape and size of all nanoparticulate materials were examined using transmission electron microscopy, and the specific surface areas (SSA) were measured by nitrogen adsorption on a Micromeritics Tristar (Gosford, NSW, Australia) at 77K using

the Brunauer-Emmet-Teller (BET) method after outgassing at 150°C for 1 hour. A 50 mM 3-[Tris(hydroxymethyl)methylamino]-1-propanesulfonic acid (TAPS, Fluka, Buchs, Switzerland) buffer adjusted to pH 9 was used to assess the effect of pH.

Calcium hydroxide ($Ca(OH)_2$, puriss., Riedel-de Haen Chemicals, Hannover, Germany) was obtained from a commercial source.

Assessment of alkaline capacity

Here and in all further experiments, 1:20 (wt:vol) suspensions (corresponding to 50 mg/mL) of the materials in unbuffered physiologic saline (0.9% NaCl) were used. To visualize the alkaline capacity of these suspensions, they were potentiometrically titrated using a 1 M HCl solution.

Raman spectroscopy

Raman spectroscopy (Bruker FRA 106/S and Equinox 55, Ettlingen, Germany) was carried out in 180° backscatter mode for dried powders after 7 and 30 days immersion in saline at room temperature and no fluid exchange. The slurries were centrifuged three times for 5 minutes at 13,000 rpm and washed intermediately with deionized water, dried overnight in a vacuum oven (Salvis, Luzern, Switzerland) at 250 mbar at 70° C with a constant nitrogen flow and ground by hand in a mortar. The intensity of the phosphate (P-O) and carbonate (C-O) peaks at 960 cm^{-1} and 1080 cm^{-1} (Rehman *et al.* 1994) in comparison to as prepared material are a qualitative argument for the degree of carbonated hydroxyapatite formation on the surface, the main contributor in mineralization (Notingher *et al.* 2002).

Assessment of antimicrobial efficacy

Standardized bovine dentine disks were prepared as follows: cylinders with a diameter of 7 mm were cut from the crowns of extracted bovine front teeth using a trephine bur. Subsequently, a disk with a thickness of 0.8 mm was cut from the dentine section of the cylinder using a saw microtome (SP 1600, Leica, Wetzlar, Germany). Disks were then immersed in an excess of a 2% NaOCl solution for 10 min at room temperature to dissolve organic remnants and possibly present microorganisms. The disks were then transferred into 2% sodium thiosulfate to stop the hypochlorite action. Subsequently, the disks were immersed in tryptic soy broth (TSB, Oxoid, Basingstoke, UK) and autoclaved. *Enterococcus faecalis* ATCC 29212 (stock stored at -70°C) was cultured in TSB overnight at 37°C (TSB + 10% glycerol vol:vol). The disks, which had been autoclaved in TSB, were then transferred to sterile 24-well plates. Each well contained 1.6 mL of sterile TSB. Each of these wells was inoculated with 100 μL of the TSB containing the *Enterococci* and incubated for 24 h at 37°C in ambient air. Subsequently, the disks were dipped three times in three separate wells containing 0.9% saline to wash away loosely adherent bacteria. The disks were then transferred into microcentrifugation tubes containing 1:20 (wt:vol) suspensions of test or control materials in saline and incubated for 1 h, 1 d, or one 1 w. Incubation of disks in sterile saline was used as the positive control treatment.

For the harvesting of the biofilm, the disks were gently dipped in sterile saline to get rid of excess medicament. They were then transferred into 1 mL of saline, vortexed vigorously for 2 min, and finally gently ultrasonicated (Vibracell, Sonics & Materials,

Newtown, CT, USA) at 20 W in an ice bath for 5 sec (Guggenheim *et al.* 2001). The suspensions were kept on ice, serially diluted and cultured on tryptic soy agar (TSA, Oxoid). The plates were incubated at 37°C for 48 h. Colonies were counted under a stereo dissecting microscope, and colony forming units (CFU) were calculated. The purity of the cultures was verified by visual inspection of the colony morphology as well as Gram staining.

Analysis of supernatants

Immediately after the bacteria were harvested as described above, the microcentrifuge tubes containing the test or control suspensions were centrifuged at 13,000 x g for 30 min at 4°C. Subsequently, 500 µL of the supernatant was transferred into separate tubes and stored at -20°C until further analysis. Osmolarity was assessed in an osmometer (One-Ten, Fiske, Needham Heights, MA); pH was determined using a calibrated microelectrode (Metrohm). Ca and Si concentrations in solution were measured by using an atomic absorption spectrophotometer (Model 2380, Perkin-Elmer, Norwalk, CT) as described earlier (Zehnder *et al.* 2006b).

Scanning electron microscopy

Specimens (n = 4 per group) were washed in saline and fixed at room temperature in 2% glutaraldehyde for 30 min. Thereafter, specimens were dipped in saline, dehydrated in an ascending ethanol series and dried using the critical point method in a CPD 030 device (BAL-TEC, Balzers, Liechtenstein). Dry specimens were glued to scanning electron microscopy stubs and coated with gold in a sputter coater (SCD-500,

BAL-TEC). They were examined in a scanning electron microscope (Zeiss Supra 50 VP) at an accelerating voltage of 20 kV detecting secondary electrons.

Data presentation

Numerical data from triplicate experiments are presented as means and standard deviations.

Results

Material properties

Flame spray synthesis allowed the direct and dry synthesis of bioactive glasses with varying composition and in the form of nanoparticles. The specific surface areas (SSA) measured by nitrogen adsorption were 65, 72, and 147 m^2/g for 28S5, 45S5 and 77S, respectively (Table 1). In comparison, the flame-derived amorphous TCP nanoparticles had a SSA of 80 m^2/g. 77S, which contained no sodium, showed well defined, spherically shaped nanoparticles. In contrast, 28S5 displayed highly agglomerated particles with visible sinter necks as observed by transmission electron microscopy (images not shown).

pH induction and buffer capacity

When suspended in unbuffered saline solution, 28S5 and 45S5 induced similar pH values to calcium hydroxide. However, their potential to keep the pH at this level upon addition of a hydrochloric acid was lower for the glasses than for the calcium hydroxide

(Fig.1). In contrast, the initial pH with the sodium-free glass 77S was <10, and no notable alkaline capacity was observed.

Mineralization

The ability of the nanoparticulate glasses and TCP to form a crystalline apatite layer on the surface of the material, *i.e.* the *in vitro* mineralization, was investigated by Raman spectroscopy. When placed in physiological saline solution for 7 and 30 days, all glasses were able to form an apatite layer on their surface (Fig. 2). TCP showed by far the highest apatite peak (Fig. 2), indicating the highest mineralization capability. This was in line with SEM images of treated bovine dentine discs which showed substantial crystal formation not only observed on the dentine, but also on bacteria (Fig. 3, bottom right).

Antimicrobial efficacy

The disinfection properties of the different bioactive glasses and reference materials are shown in Fig. 4 displaying the $\log_{10}$ CFU values for the *Enterococci* after the treatment with the different medicaments for 1hour, 1day and 1week. Calcium hydroxide had a very fast effect and resulted in a complete killing of the bacteria within one hour. At conditions used in this study, 45S5 nanoparticles were able to kill all bacteria of the biofilm within one day. The effect of the 28S5 composition was comparable, although the results showed a larger standard deviation. When treated with 77S, some bacteria were able to survive for one day, but after one week this treatment also left no viable cells. No clear reduction in viability was observed for TCP, as well as for a buffered solution of pH 9.

In order to get a picture of the dentine surface with the adherent bacteria, the samples were investigated by SEM after 1 week of incubation. Without treatment, the bacteria were spread out on the dentine surface, whereas a treatment using calcium hydroxide did not only kill and lyse all bacteria, but also attacked and eroded the dentine (Fig. 3, top right), which did not occur in other treatments. The surface of TCP treated dentine showed deposition of mineral and partial encapsulation of the bacteria by these deposits (Fig 3, bottom right). Apatite mineral deposition was not visible for the bioactive glass treated surfaces by SEM.

Ion concentrations in supernatants

Osmolarity measurements revealed that values did not change significantly over the course of one week. The results for bioactive glasses 28S5, 45S5 and 77S were 760±60 mOsm, 560±20 mOsm and 300±20 mOsm respectively. For TCP values of 290±10 mOsm, calcium hydroxide 330±10 mOsm and for unbuffered saline solution 300±10 mOsm were reached. All values are significantly lower than the by *E. faecalis* experimentally tolerated osmolarity of 1800 mOsm described in the literature (Zehnder *et al.* 2006b).

After exposure 28S5 bioactive glass supernatants showed high silicon concentrations in solution in the range of 1000 ppm for the three time points (Fig. 5). Si concentrations for 45S5 glass were in the same range, however, and increase of dissolved Si was observed over time. On the other hand 77S showed minimal amounts of Si in solution. No dissolved silica species in solution were observed for all other materials. Measurements on the calcium concentrations in solution showed low to no Ca for the

28S5 and 45S5 glasses whereas 77S and TCP showed concentrations in the range of 100 to 200 ppm. Calcium hydroxide effected concentrations of 1000 ppm Ca in solution.

Discussion

This study showed that the pH generated in an aqueous environment by silica-containing bioactive glasses is affected by the sodium content in the glass. Furthermore, the high pH in the environment of sodium-containing bioactive glasses is the main material property that conveys the antimicrobial effect. There is, however, also an additional effect with a slower onset, which is related to the sustained release of silica and/or calcium phosphate species from the glass. Calcification of microbiota adhering to dentine, on the other hand, whilst it did occur, did not affect the viability of the *E. faecalis* type strain under the current conditions.

As already observed within a previous study, nanoparticulate bioactive glass 45S5 is a potent disinfectant (Waltimo *et al.* 2007). The main purpose of this study was to further elucidate the antimicrobial effect of bioactive materials such as the glasses under investigation. A relatively large amount of data is reported in this communication. Consequently, the individual findings are discussed in the following subchapters.

Physical properties of the materials under investigation

Flame-spray synthesis is a dry, one-step synthesis method to prepare complex materials (Stark *et al.* 2002) and specifically tailor the composition of such materials such as the bioactive glasses for biomedical applications. For the different ultrafine bioactive glasses described here the surface areas were reduced with increasing sodium content,

which also had an effect on the morphology of the material. It is a well known fact that the addition of sodium into a silica glass matrix is decreasing its melting point. It can therefore be expected that a variation of sodium content in the glass has an effect on the product properties when flame spray synthesis is applied since sintering can occur in the flame resulting in a decrease in surface area. Both sodium concentration and specific surface area affect the solubility of the glass when placed in an aqueous environment. However, the effect of the surface area of the material was not further investigated in the current study.

Monospecies *E. faecalis* biofilm

The model used in this study was developed during a larger body of work on a multi-species biofilm on dentine (Waltimo *et al.*, International Endodontic Journal, submitted). As observed during that study, adhesion to dentine is the main factor that increases the resistance of the *E. faecalis* type strain to alkaline biocides. For simplicity reasons, we therefore chose to use a monospecies biofilm model in the current investigation. However, it must be conceded that this model does not necessarily reflect the clinical reality, in that infection of accessory canals and the presence of necrotic tissue are not mimicked. Furthermore, the medication to dentine ratio does not reflect the situation in the root canal. Nevertheless, the high efficacy of calcium hydroxide against *E. faecalis* was again proven in this study, and these results are comparable to *ex vivo* and clinical observations in human teeth (Sirén *et al.* 2004; Zehnder *et al.* 2006a).

Influence of pH on antimicrobial effect

The disinfection properties of calcium hydroxide are believed to result from a mere increase in pH which is not tolerated by microbiota (Proell 1949). This treatment effects lysing of bacterial cells. Under the investigated powder to liquid ratios of this study the pH caused by bioactive glasses 28S5 and 45S5 was similar to that of calcium hydroxide. However, the buffer capacity of these glasses was substantially lower. This might be the reason why these bioactive glasses showed a slower onset of the antimicrobial effect, although their killing of adherent *E. faecalis* was still potent, clearing all bacteria after 24 hours of incubation. This antimicrobial potency could be sufficient for their proposed application as a topical antiseptic. Most interestingly, 77S was able to disinfect the dentine surface within 1 week with a by far lower pH induction of around 9. We used TAPS buffer adjusted at pH 9 to investigate if such a buffer results in the same reduction of viable bacteria after 1 week. However, a system with saline solution buffered at pH 9 did not result in the same antimicrobial efficacy but showed no reduction at all. This clearly indicates that the effect of 77S is not related to a mere pH effect, but might be related to an effect of liberated ions or mineralization.

Influence of ion release

On the SEM images of bacteria on the dentine surface, no apparent mineralization has occurred for treatments with the different bioactive glasses. We can therefore attribute the antimicrobial effect of 77S bioactive glass to the release of ions leaching out of the glass which consequently affects the metabolism of the bacteria. On 28S5 and 45S5 treated dentine no bacteria were found on the surface, indicating that they were

lysed. In contrast, with the 77S treatment bacteria were found on the dentine surface, which were not able to divide anymore under the current surrounding conditions.

Which ionic species or combination thereof account for the secondary antimicrobial bioactive glass effect, however, needs to be elucidated in a further study and was beyond the scope of the present work. This issue is fairly complex, as the pH influences the type and amount of ionic species in bioactive glass dissolution (Fig. 6).

Influence of mineralization

All bioactive glasses were able to form an apatite layer on the surface of the dentine, which is indicated by the *in vitro* bioactivity observed by Raman spectroscopy (Fig. 2). This mineral deposition was speculated to have an effect on the viability of the cells by mechanically encapsulating the bacteria (Zehnder *et al.* 2006a). However, this deposition of a layer of apatite mineral is generally very thin. As a reference material amorphous TCP was used, which is known to have an even higher mineralization potential than the bioactive glasses (Brunner *et al.* 2007; Loher *et al.* 2006), but does not cause a raise in pH. Crystalline structures on the surface and around bacteria proved this phenomenon as observed by SEM (Fig. 3, bottom right panel). However, the calcium phosphate deposits did not decrease the viability of the enterococci on the dentine surface attesting the mineralization a minor influence on the antimicrobial properties. Further studies need to elucidate the ideal glass preparation and composition that can match or surpass the antimicrobial effect of calcium hydroxide in the root canal without causing the damage to the dentine substrate that calcium hydroxide provokes.

Conclusion

This study highlighted the importance of sodium and the thus-resulting alkaline environment created by bioactive glasses on their antimicrobial effect. Furthermore, a second antimicrobial effect of bioactive glasses in the absence of sodium can be attributed to their continuous release of ionic species in aqueous suspensions.

Acknowledgements

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Tables

Table 1 Nominal composition of the different nanoparticulate materials in weight percent and their specific surface area (SSA).

	SiO_2	Na_2O	CaO	P_2O_5	SSA [m^2/g]
Bioactive glass 28S5	27.5	42.0	24.5	6.0	65
Bioactive glass 45S5	45.0	24.5	24.5	6.0	71
Bioactive glass 77S	79.7	-	16.3	4.0	147
Tricalcium phosphate	-	-	54.2	45.8	80

Figure Captions

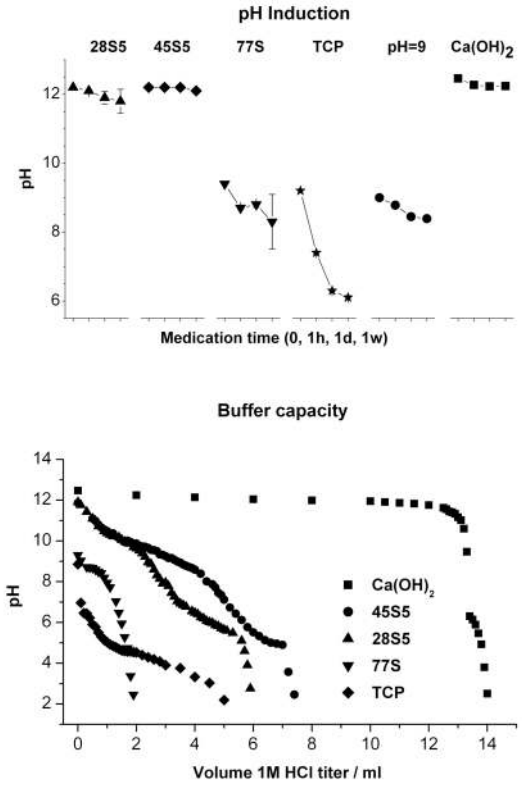

Figure 1 pH values caused by the different materials when immersed in saline solution (top). Ultrafine 28S5 and 45S5 particles effected similar pH values than calcium hydroxide. Buffer capacity of the different materials when titrated with 1M hydrochloric acid (bottom).

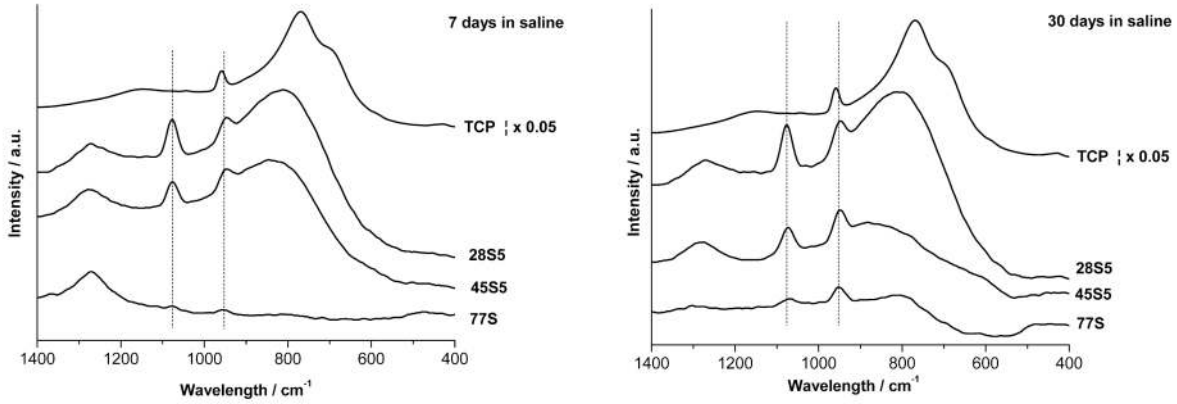

Figure 2 Raman spectroscopy of the materials after incubation in saline for 7 (left) and 30 days (right). The peaks attributed to carbonated hydroxyapatite are highlighted.

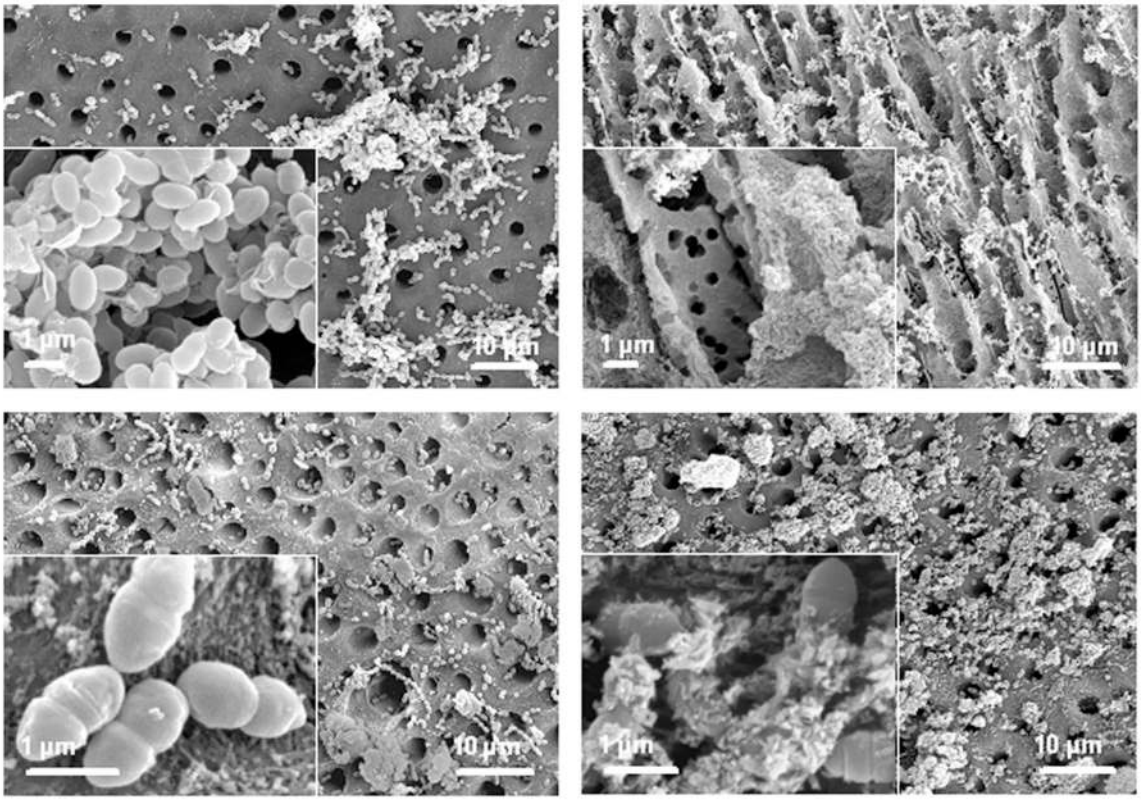

Figure 3 SEM images of dentine surfaces after treatments for 1 week. Saline did not reduce the amount of adhering bacteria (top left). Calcium hydroxide left no bacteria on the dentin but also damaged the dentin surface (top right). Some bacteria on dentin treated with ultrafine 77S were visible but showed no viability (see Fig 4). The dentine surface is intact (bottom left). Nanoparticulate TCP caused precipitate formation on the dentine surface partially covering bacteria (bottom right).

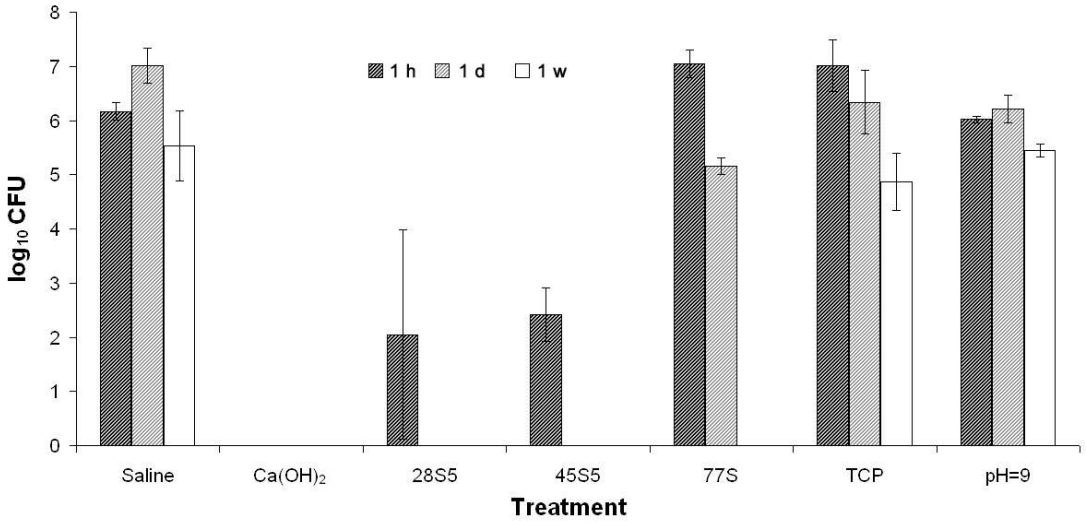

Figure 4 Viable *E. faecalis* bacteria from the dentine surfaces after treatments for 1 hour, day and week in saline solution (mean $\log_{10}$ CFU values (n=3) and standard deviation).

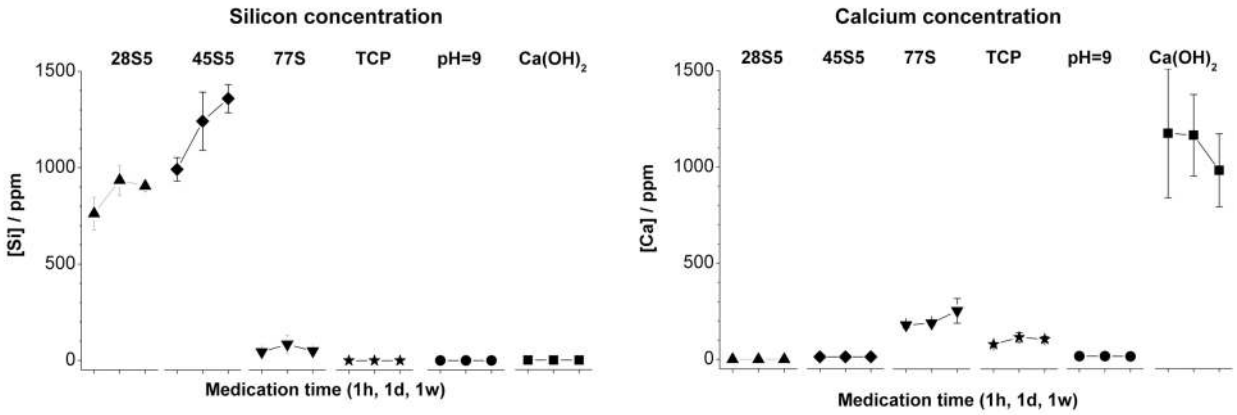

Figure 5 Concentrations of silicon and calcium in the supernatants after medication. Mean values (n=3) and standard deviation are shown.

Figure Captions

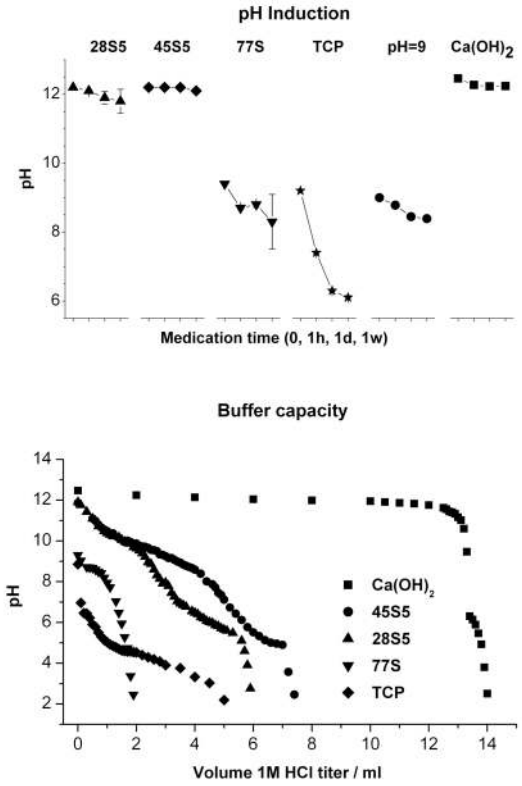

Figure 1 pH values caused by the different materials when immersed in saline solution (top). Ultrafine 28S5 and 45S5 particles effected similar pH values than calcium hydroxide. Buffer capacity of the different materials when titrated with 1M hydrochloric acid (bottom).

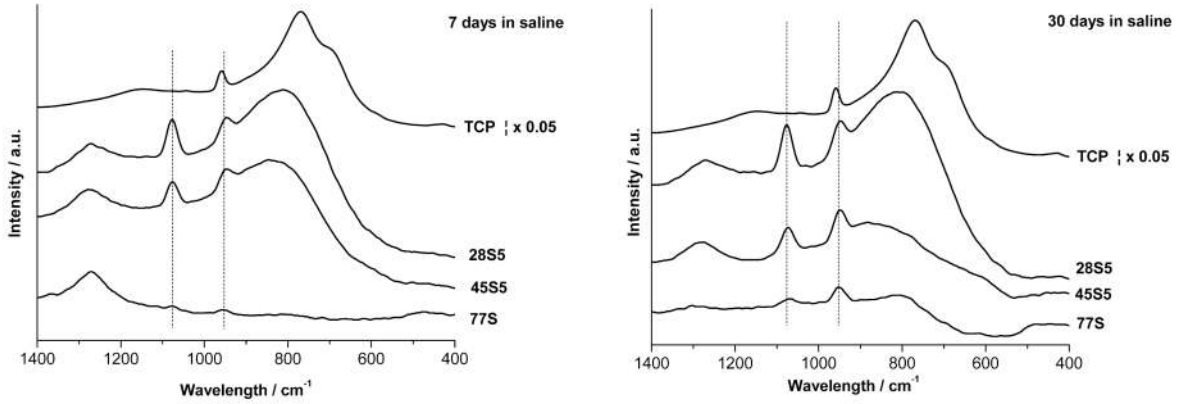

Figure 2 Raman spectroscopy of the materials after incubation in saline for 7 (left) and 30 days (right). The peaks attributed to carbonated hydroxyapatite are highlighted.

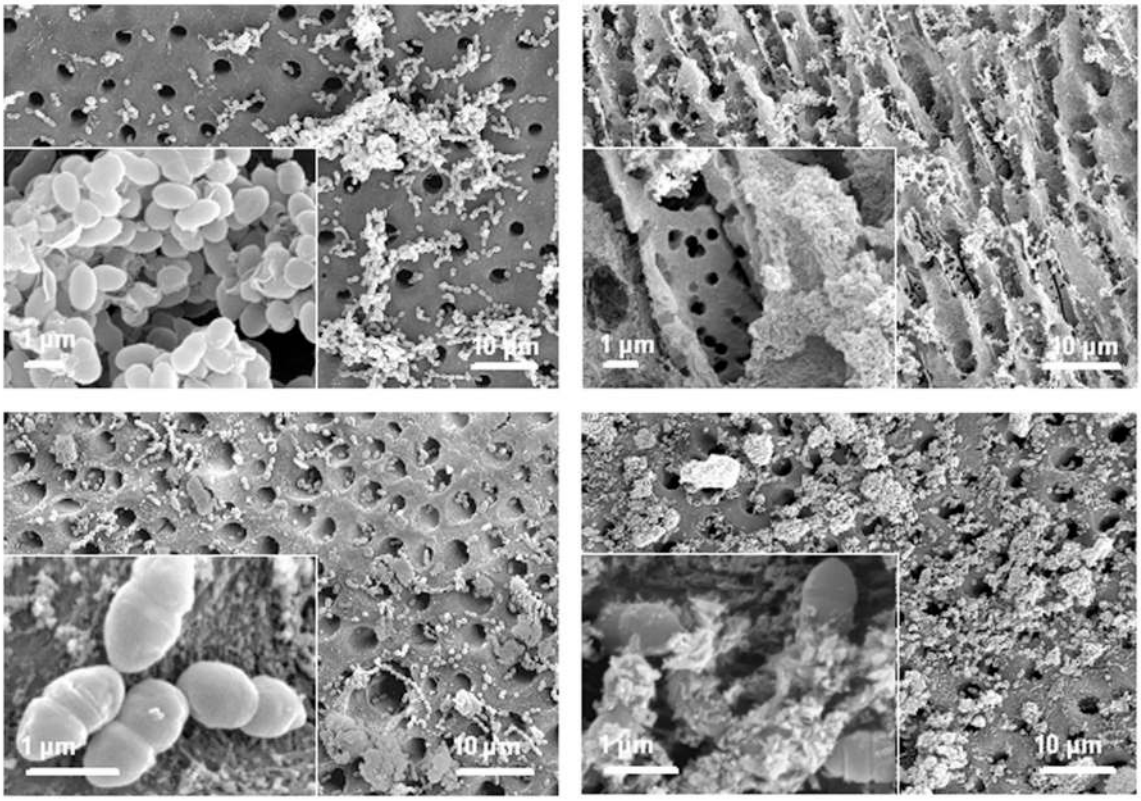

Figure 3 SEM images of dentine surfaces after treatments for 1 week. Saline did not reduce the amount of adhering bacteria (top left). Calcium hydroxide left no bacteria on the dentin but also damaged the dentin surface (top right). Some bacteria on dentin treated with ultrafine 77S were visible but showed no viability (see Fig 4). The dentine surface is intact (bottom left). Nanoparticulate TCP caused precipitate formation on the dentine surface partially covering bacteria (bottom right).

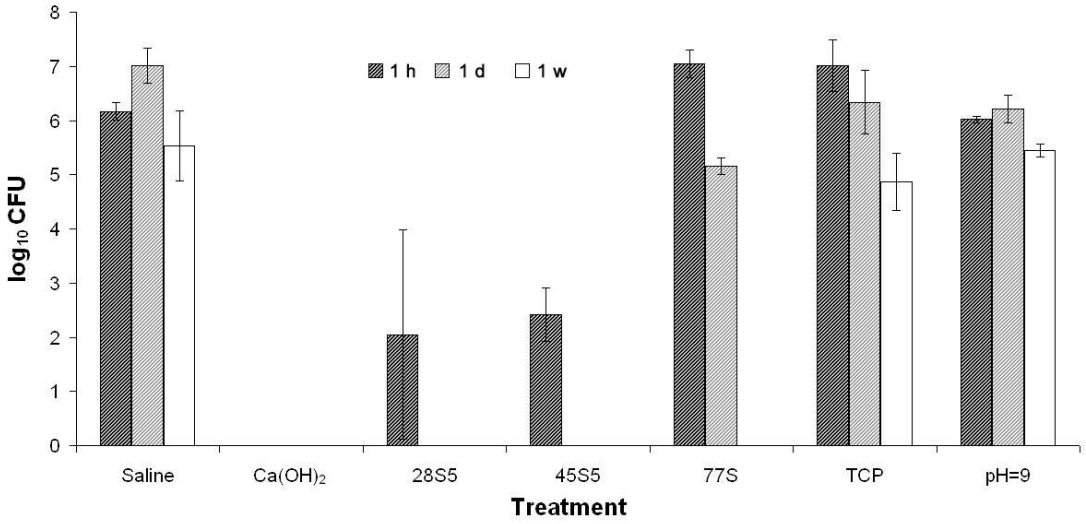

Figure 4 Viable *E. faecalis* bacteria from the dentine surfaces after treatments for 1 hour, day and week in saline solution (mean $\log_{10}$ CFU values (n=3) and standard deviation).

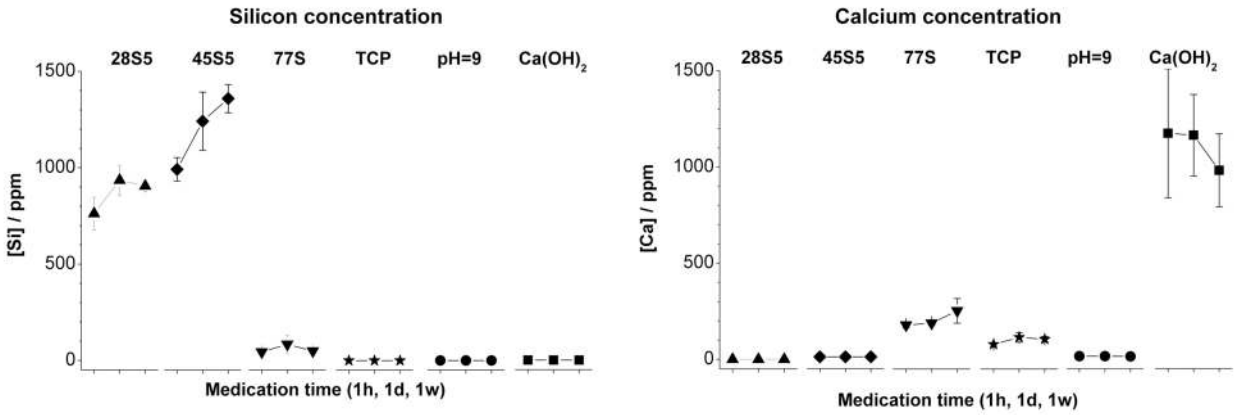

Figure 5 Concentrations of silicon and calcium in the supernatants after medication. Mean values (n=3) and standard deviation are shown.

Tables

Table 1 Nominal composition of the different nanoparticulate materials in weight percent and their specific surface area (SSA).

	SiO_2	Na_2O	CaO	P_2O_5	SSA [m^2/g]
Bioactive glass 28S5	27.5	42.0	24.5	6.0	65
Bioactive glass 45S5	45.0	24.5	24.5	6.0	71
Bioactive glass 77S	79.7	-	16.3	4.0	147
Tricalcium phosphate	-	-	54.2	45.8	80