Mineralization of dentin induced by treatment with bioactive glass S53P4 in vitro

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Dentin hypersensitivity can be managed to occlude dentin tubules, but none of the agents used are components of natural dentin. Using a calcium phosphate precipitation (CPP) method, dentin tubules can be occluded with a calcium phosphate (CaP) layer similar to the major inorganic component of dentin. The CPP method utilizes acidic pH conditions, such as etching of dentin, over the course of several dental treatments. A gentler method can be used to produce a CaP layer on the surface of dentin. By treating with bioactive glass S53P4 (BAG), or regular commercial glass (CG), mineralization occurs in physiologically neutral solutions such as simulated body fluid (SBF) and remineralization solution (RMS). After a short period of immersion, silica is dissolved from both types of glass, but the amount of silica released is much greater from BAG than from CG. The dissolved silica is adsorbed on the surface of dentin during the pretreatment procedure and enhances the mineralization of dentin in SBF. After 14 days' mineralization the dentin is fully covered by the CaP layer, but after 14 days' immersion in RMS decalcification of the dentin occurs. Pretreatment with BAG decreases the degree of decalcification of dentin during the mineralization process. These findings suggest that bioactive glass S53P4 can be used as a therapeutic material for mineralization of dentin and its tubules in a physiological environment. ☐ *Biomimetic; calcium phosphate; dentin; remineralization; silica*

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Dentin is the mineralized tissue located between the crown enamel or root cementum and the pulp of the tooth. It contains a large number of cylindrical channels, i.e. dentinal tubules that extend from the peripheral dentin into the pulp. The cervical roots of the teeth are usually covered by gingiva, but, with aging, excessive brushing, or periodontal therapy, the gingiva may recede, thus exposing the cervical cementum. However, exposure of the root surface renders it prone to cervical caries and is often the pragmatic reason for erosion of the cervical cementum and dentin hypersensitivity (1).

The cornerstones of prevention of different types of caries lesions are daily diet and cleaning, fluorides, stimulation of salivary flow (xylitol) and occasionally the use of remineralizing solutions (2). The clinical value of these remineralization solutions, however, is a matter of debate. (3)

Dentin hypersensitivity is managed by agents such as potassium oxalate (4) and ferric oxalate (5), and by resins (6) reported to occlude dentinal tubules or decrease their diameter. The desensitizing effect of those products is weak (7) or relatively short-lived (8) in the oral environment and none has shown superior efficiency. Furthermore, the occluding materials formed by these treatments are not components of the natural dentin. To overcome this, a calcium phosphate precipitation (CPP) (9) method has been developed where the dentin is treated with an acidic calcium phosphate (CaP) solution which is then neutralized with a basic post-treatment solution. With this treatment the dentin tubules are occluded with a CaP layer similar to the major inorganic component of dentin and bone. However, the CPP method utilizes extreme acidic pH conditions that could harm the dentin during treatment. Similar acidic pH conditions are actually used in the etching of dentin during several dental practices. In order to avoid these drawbacks gentler treatments are required.

The recently developed biomimetic calcification method makes use of simulated body fluid (SBF) in neutral pH, resulting in the formation of bone-like hydroxyapatite on even organic polymers (10–12). With this method the substrate is first brought into contact with an SBF solution containing CaO-SiO_2-based glass particles to induce CaP nucleation. It has been suggested that the silica released from the glass particles adsorbs on the substrate, so providing sites for heterogeneous CaP nucleation. Once nucleated it will spontaneously grow in a solution supersaturated with respect to the apatite to form a bone-like apatite layer. It has also been reported that silica promotes the mineralization of dentin (13, 14) by a mechanism based on the condensation of silicic acid to oligomers (15).

The objective of this in vitro work was to study the use of bioactive glass and the biomimetic method on dentin mineralization. Bioactive glass S53P4 (BAG), composed of SiO_2, Na_2O, CaO, and P_2O_5, was used as the calcium and silica source and regular commercial glass as control (CG). After pretreatment, the dentin samples were kept in SBF or in a remineralization solution (RMS) to complete the

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mineralization process. The composition and morphology of the treated dentin were studied using scanning electron microscopy and energy-dispersive X-ray analysis (SEM-EDX). The surface of dentin and the adsorbed silica was monitored using X-ray photoelectron spectrometry (XPS).

Table 1. Preparation of mineralization solutions A and B

	Stock 1 (mL)	Stock 2 (mL)	Stock 3 (mL)	H_2O (mL)
Mineralization solution A	100	–	100	800
Mineralization solution B	–	100	100	800

Materials and methods

Dentin disks

Extracted human 3rd molars were cleaned with hand instruments to remove the soft tissue. Each tooth was cut along its horizontal axis just below the dentin-enamel junction using a water-cooled low speed diamond saw (Struers, Accutom-50). Four 200-μm-thick disks prepared from each tooth were then cleaned with 0.5% NaOCl at room temperature for 5 min. The surface areas of dentin disks were measured with light microscopy (Wild M3Z Kombistereo, Switzerland) equipped Microscale software (Digithurst, Royton, UK). Before the experiment, the specimens were weighed to constant weight and then washed with an excess of ion-exchanged water and 70% ethanol (EtOH) for 20 min to sterilize the disk.

Glasses

Two different types of glass were used for the experiments: bioactive glass S53P4 (BAG; 23 wt% Na_2O, 20 wt% CaO, 4 wt% P_2O_5, 53 wt% SiO_2, Abmin Technologies Ltd., Turku, Finland) and control glass (CG, Iittala Finland). Both types of glass were used as powder with a particle size <45 μm.

Solutions

Tris buffer (50 mM) used for silica dissolution studies was prepared by dissolving TRIZMA® Pre-set Crystals (Sigma) in ion-exchanged water. The final pH of the solution was 7.4 (37°C).

The mineral formation was studied in vitro using simulated body fluid (SBF) and commercially available remineralization solution (RMS). The SBF was prepared by dissolving reagent chemicals of NaCl, $NaHCO_3$, KCl, $K_2HPO_4 \times 3\ H_2O$, $MgCl_2 \times 6\ H_2O$, $CaCl_2$ and Na_2SO_4 in ion-exchanged water. The solution was buffered at physiological pH 7.4 (37°C) with tris(hydroxymethyl aminomethane) and hydrochloric acid. The ion concentrations of SBF (Na^+142.0, K^+5.0, Mg^{2+}1.5, Ca^{2+}2.5, Cl^-147.8, HCO_3^- 4.2, HPO_4^{2-} 1.0, SO_4^{2-} 0.5 mM) are nearly equal to that of human plasma (16).

The RMS was prepared by mixing 3 different stock solutions. Stock solution 1 was prepared by dissolving $CaCO_3$ (100 mmol) in 2 M HCl (100 mL). After dissolution, ion-exchanged water was added and the solution was buffered with 0.5 M KOH at pH 4.0. Stock solution 2 was prepared by dissolving NaH_2PO_4 (60 mmol) in ion-exchanged water and adjusting the pH to 7.5. Finally, stock solution 3 was prepared by dissolving the reagents of NaCl and NaF (1.5 mmol and 50 ppm, respectively) in ion-exchanged water. Mineralization solutions A and B were prepared by mixing stock solutions as shown in Table 1. The final remineralization solution was prepared by mixing the mineralization solutions A and B 1:1 (v:v). The pH of the final solution was 7.4 (37°C). All solutions were either filtered through a membrane with 0.45 μm pores (PALL, German laboratory) or autoclaved (121°C, 15 min) before use.

Silica release analysis

The dissolution of silica (SiO_2) from both glasses was studied in Tris buffer solution as a function of immersion time up to 24 h. Tris buffer was used in order to avoid the possible interfering of phosphate ions during measurement. Silica concentrations were analyzed using the molybdenum blue method with UV-VIS spectrophotometer (Shimadzu UV-1601). The analysis is based on the reduction of 1-amino-2-naphthol-4-sulfonic acid (17).

Biomineralization process

Biomimetic mineralization of the dentin disks was done as described earlier (10–12) with minor modifications. First, the disks were pretreated at 37°C for 24 h in 30 mL of either SBF or RMS solutions. These pretreatment solutions were supplemented with 15 mg of BAG or CG (silica sources). Pure non-supplemented solutions were used as controls. Second, the SBF pretreated dentin disks were moved to another sample tube containing pure SBF and the RMS pretreated disks to tubes containing pure RMS, respectively. The mineral growth in SBF and RMS (30 mL) was conducted for 14 days at 37°C under continuous gentle shaking (60 strokes/min) and the solutions were changed every second day. The experimental design is presented schematically in Fig. 1. After 14 days the dentin disks were washed in ion-exchanged water and dried at 45°C to constant weight. The dentin disks were weighed before and after mineralization.

Surface analysis

Morphology and chemical analyses of the sample surfaces were performed using scanning electron microscopy and energy-dispersive X-ray analysis (SEM-EDX, Stereoscan 360 Cambridge). The surfaces of the specimens were coated with carbon film for SEM-EDX monitoring.

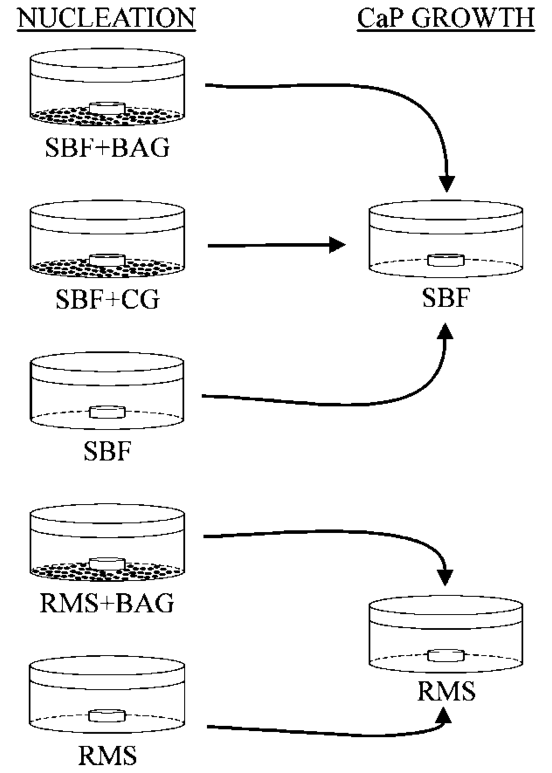

Fig. 1. Schematic picture of the dentin mineralization procedure. After 24 h nucleation the dentin was moved to another tube, where CaP growth occurs.

Further information of the chemical nature of the samples was obtained by the surface sensitive X-ray photoelectron spectroscopy (XPS, Perkin Elmer PHI 5400 ESCA System Spectrometer) using the MgK_α X-ray ($\lambda = 1253.6$ eV) source. The UNIFITTU (version 2.1) software was used for peak fitting and quantitative chemical analysis. The high-resolution spectra were charge compensated by setting the binding energy (BE) of the C(1s) contamination peak to 284.6 eV.

Results

Release and adsorption of silica

Before conducting the biomimetic mineralization process, the release rate of silica was determined from both glasses in Tris buffer. Already after 15 min of immersion in Tris buffer, silica was dissolved from both glasses, but the amount released was about 200 times greater from BAG than from CG (Fig. 2). After 8 h of immersion, the amount of silica dissolved from BAG reached saturation point, whereas CG continued to release silica at a low but constant speed for the whole experiment, i.e. a period of 24 h.

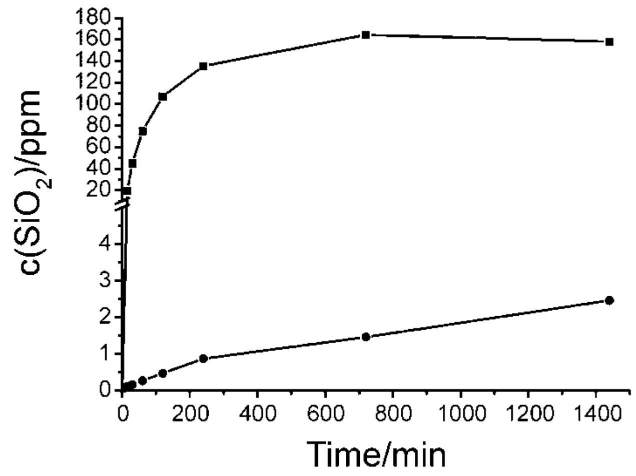

Fig. 2. Dissolution of silica from BAG (■) and CG (●) in Tris buffer for 24 h.

Biomineralization of dentin

After pretreatment of the dentin with glasses, SEM-EDX analysis showed that the dentin surface was observed composed of calcium and phosphorus but hardly any silicon. More surface-sensitive XPS measurements were therefore applied. In addition to calcium and phosphorus, silicon (Si) was observed in SBF with BAG pretreated samples corresponding to expectations from the silica dissolution data. The relative amount of silicon increased from 0.5 at% to 9.5 at% after 15 min and 24 h immersion, respectively. Peak fitting using UNIFITTU software demonstrated (Fig. 3) that the Si_{2p} peak results from two

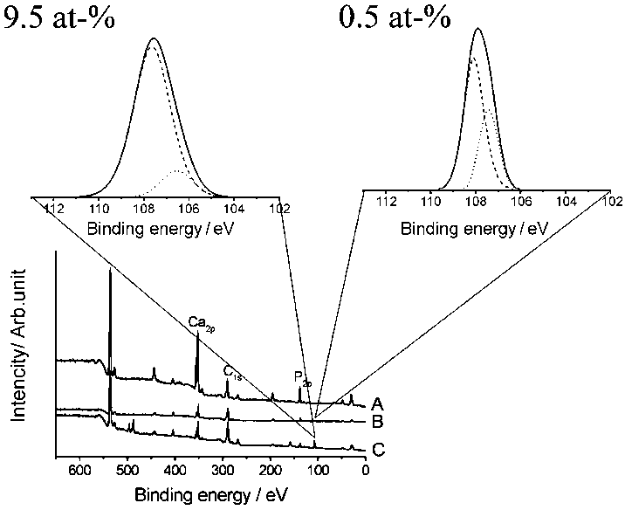

Fig. 3. Silica adsorbtion to dentin surface. XPS patterns of the surface of dentine before (A) and after 15 min (B) and 24 h (C) immersion with BAG. High-resolution Si_{2p} spectra of the surface of dentin slide after 15 min and 24 h immersion with BAG. Major peaks were SiO_2 and minor peaks SiOH.

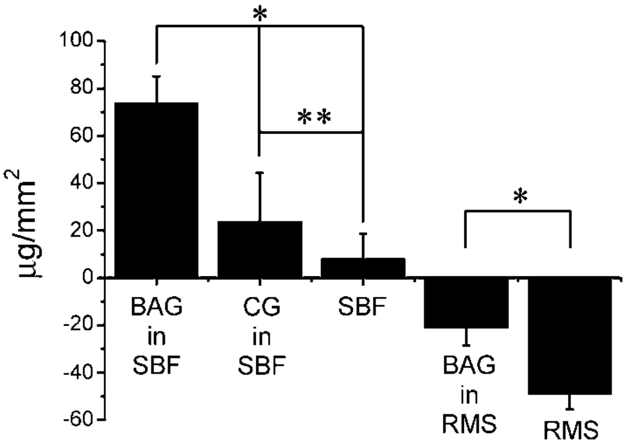

Fig. 4. Formation of CaP. The weight of CaP per surface area of dentin slide (μg/mm^2) after 14 days' mineralization (* $P < 0.001$, ** $P < 0.05$).

different Si species having binding energies (BEs) ~103.2 eV and ~101.9 eV resulting from SiO_2 and SiOH, respectively. The relative amount of SiO_2 increased during immersion time from 64.5 at% at 15 min to 87.3 at% at 24 h of the total silicon amount.

Weight changes of the dentin disks after the entire mineralization procedure corresponded either with CaP formation (if increased) or decalcification (if decreased) of the dentin. It was found that pretreatment with BAG in SBF induced CaP formation that was over 2 times greater (73.6 ± 11.5 μg/mm^2) than treatment with CG (23.7 ± 20.6 μg/mm^2) and nearly 10 times greater than the control (7.9 ± 10.7 μg/mm^2) (Fig. 4 and Table 2). The 14 days immersion in RMS, however, decreased the weight of the dentin disks. The weight loss was significantly ($P < 0.001$) greater when the disks were incubated in RMS without BAG pretreatment (–49.1 ± 6.5 μg/mm^2) than with BAG pretreatment (−21.0 ± 7.4 μg/mm^2) in RMS. Fig. 4 shows significant differences between the mean amount of CaP formation after mineralization. Significant differences ($P < 0.001$) were found between treatment with BAG and CG, as well as BAG and control. The difference in the amount of CaP on dentin treated with CG and control was not statistically significant ($P < 0.05$).

Dentin mineralization with pretreatment of BAG in SBF produced a dense and uniform CaP layer on the dentin surface, and all the dentin tubules were covered (Fig. 5a, b). It can be seen from the SEM images that the reasonable CaP layer was not formed when mineralization occurred with pretreatment with CG or without pretreatments with glasses (Fig. 5c). Although some of the dentin tubules were partly sealed with CaP, there were still open tubules. The mineralization in RMS produced a rough and fluffy CaP layer on the dentin surface covering the dentin tubules (Fig. 5d, e), although the weight of the dentin disks was reduced during the experiment. Control dentin with open tubules is shown in Fig. 5f. The Ca/P ratios obtained from EDX measurements of the precipitated material varied from 1.75 to 1.85.

Table 2. Amounts of Ca/P per mm^2 and Ca/P ration of mineralized dentins and pure dentin

	μg/mm^2 (Mean ± *s*)	Ca/P ratio
BAG in SBF	73.6 ± 11.5	1.79
CG in SBF	23.7 ± 20.6	1.83
BAG in RMS	−21.0 ± 7.4	1.83
RMS	−49.1 ± 6.5	1.75
SBF	7.9 ± 10.7	1.78
Dentin/enamel	–	1.68/1.79

s = standard deviation.

Discussion

Although both glasses (BAG and CG) are soluble, only S53P4 is bioactive by definition, because it promotes CaP formation on the silica-rich surface of the glass itself (18) and on materials brought into contact with it (10–12). BAGs are therefore considered efficient silica and calcium sources for biomineralization processes. Technical glasses such as used in this study are not bioactive because they cannot form either a Si-rich sublayer or a CaP layer on their surface. Over 3 times more silica dissolves from BAG after 15 min of immersion in SBF than from CG during 24 h immersion, which suggests that within 15 min of treatment with BAG a considerable amount of silica is released to be adsorbed on the dentin. According to Damen et al. (13, 14), silica on dentin acts as a heterogenic nucleation center for CaP precipitation. In addition, BAG releases high concentrations of Ca^{2+} which increase the concentration of Ca^{2+} ions in the vicinity of the material and enhance mineralization (19).

After pretreatment with the less soluble CG, no silica was found on the dentin surface, even with surface-sensitive XPS analysis. However, pretreatment with BAG resulted in high concentrations of adsorbed silica, which increased during the immersion time (Fig. 3). Theoretically, the silica that was dissolved from CG could also adsorb to the dentin, but due to the low amount it was not detected. These results are well in line with previous reports (13, 14) showing that soluble silica adsorbs on the dentin surface and promotes mineralization. When adsorbed silica is condensed on the dentin (15), there are still free silanol groups (SiOH) that are thought to act as CaP nucleation centers (20). It is also suggested that Ca^{2+} released from the BAG increases the ionic activity product of apatite and thus promotes nucleation of CaP (21). Once the apatite nuclei are formed, they grow by consuming calcium and phosphate ions from the surrounding SBF and, perhaps, also from saliva.

In general, CaP nucleation in pure supersaturated Ca^{2+} and PO_4^{3-} solution such as simulated SBF or RMS can occur by two mechanisms: homogeneous nucleation occurs spontaneously in a solution and heterogeneous

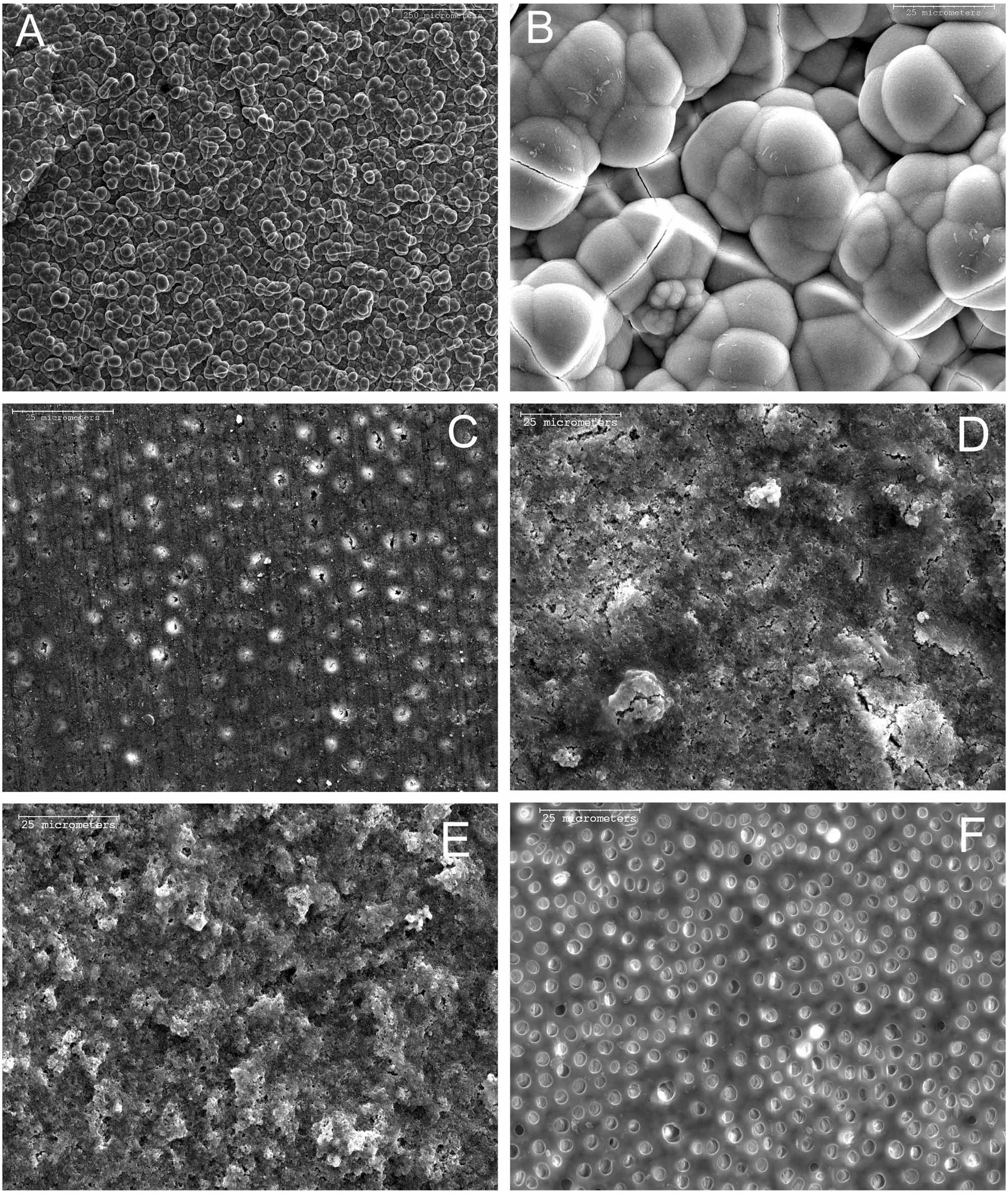

Fig. 5. Dentin surfaces after 14 days' mineralization with BAG (A and B) and without SiO_2-source in SBF (C) with open dentin tubules. Dentin surface after 14 days' mineralization with (D) and without BAG (E) in remineralization solution and unmineralized dentine slide (F). Bar = 250 μm (A), bar = 25 μm (other).

nucleation occurs on the surface of a substrate. Mineralization using BAG or CG pretreatment in SBF appears to take place through the heterogeneous nucleation mechanism; mineralization without BAG in RMS goes through the homogeneous nucleation mechanism. Treatment of dentin with BAG in SBF produces a dense and uniform CaP layer on its surface and all the dentin tubules are sealed (Fig. 5a, b). When the treatment is performed with CG or without any SiO_2 source (Fig. 5c), the CaP did not completely cover the surface and there were still plenty of open dentinal tubules. Accordingly, silica increases CaP formation and significantly shortens the induction period of CaP precipitation.

Although mineralization in RMS (Fig. 5d, e) reduced the weight of dentin disks, a rough and dense CaP layer formed on the dentin surface and sealed the dentinal tubules efficiently. Mineralization with BAG pretreatment in RMS appears to take place via both heterogeneous (on surface of dentin) and homogeneous (in solution) nucleation. Weight loss of the dentin disk was most likely caused by the low pH of RMS, which decreased from pH 7.4 to acidic pH 5.4 during the 2-day incubation period. Low pH is known to result in demineralization of cementum at pH <6.7 and enamel at pH <5.4 (22). According to the present result, treatment with bioactive glass S53P4 reduces demineralization of dentin in RMS because treatment with BAG forms more initial CaP than mineralization without BAG. It is also known that in acidic conditions dissolution of dentin mineral is greater than dissolution of pure apatitic mineral (23), which means that the pure CaP layer produced by BAG treatment may increase the resistance of dentin to its dissolution during clinical challenge (3).

It was observed from the SEM images that mineralization using different (SBF and RMS) solutions resulted in different types of CaP precipitate. The morphology of the layers formed was different even though their Ca/P ratios were similar (Table 3) and close to that of apatite (1.67). Treatment with BAG in SBF resulted in a CaP that was macroscopically fairly smooth and dense. The CaP layer itself was formed of small particles that were chemically identical and morphologically homogeneous. When CG was used as a source of SiO_2, the CaP which formed appeared similar to that obtained after BAG treatment. However, it only partly covered the dentin surface. By contrast, the CaP produced by RMS mineralization was less organized and the CaP particles that formed had a different morphology from those produced with BAG in SBF. Since the tendency of RMS solution to become acidic during storage may cause the demineralization of dentin, it is important to use fresh RMS in all mineralization treatments, especially in all home care regimens.

In conclusion, and based on these in vitro results, bioactive glass S53P4 may develop into a clinical product that could be used in the mineralization of dentin and its tubules in a physiological environment. A sufficient amount of silica is adsorbed on dentin with anionic groups (SiO^-) within a short treatment period and then act as a nucleation center for the subsequent and increased CaP formation. However, saliva is concentrated with calcium and phosphate ions, but there are also many proteins (24) which may affect CaP formation.

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