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In vitro remineralization of human dental enamel by bioactive glasses

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Abstract Different compositions of silicate-based bioactive glasses of the system SiO_2–CaO–P_2O_5 (45S, 58S, and 77S) were evaluated for teeth remineralization effect on the etched human dental enamel in simulate human oral environment. Enamel samples were subjected to demineralization in citric acid solution, then treated with different bioactive glass pastes, and finally soaked in simulated oral fluid (SOF) for 7 days before evaluation. The results indicated that enamel surface formed a homogenous and dense mineralized layer with the treatment of 45S and 58S samples. The average thickness was 4 and 2.5 μm, respectively. But 77S treatment showed a loose and uneven remineralized layer. Although the surface roughness decreased with the increase of silicon content, mechanical strength of enamel samples became less gradually. Taken together, 45S paste had the best remineralization ability, best mechanical strength, and satisfactory surface roughness. These results also indicated that the level of silicon content of bioactive glasses played a key role in dental enamel remineralization.

Introduction

Dental enamel contains over 96 wt% inorganic mineral, and the main constituent is a single calcium phosphate phase, hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$) crystallites. These hydroxyapatite crystallites are bundled together by organic molecules into organized larger-scale structures. However, the arrangement of the crystallites in the dentine inside is different from that of the outer enamel surface. In the enamel, the crystallites are larger and better aligned to each other, arranged in a direction perpendicular to the dentino-enamel junction (DEJ). Dentine is a mineralized connective tissue with an organic matrix of collagenous protein, which is porous and has microscopic channels leading to weak arrangement of apatite crystallites as compared to enamel. So the alignment of the crystallites of HAp inside-out is from disorder to order [1, 2]. When enamel is etched by all kinds of foodstuff, especially acidic beverages, the mineral phase dissolves, which is known as enamel demineralization [3]. Luckily, human oral saliva has remineralization ability at the early stage of demineralization [4, 5], deposition of minerals from saliva or oral fluid counterbalances the lost of minerals of enamel. However, when enamel is seriously demineralized, the remineralization from the oral saliva can not fully repair the demineralized teeth. Eventually the tooth structure is destroyed leading to dental caries. Up to date, this type of decay is still treated by drilling out the decayed region and inserting semi-permanent filling materials such as polymer or cement in clinic [6, 7], which is a complicated procedure for both dentists and patients. Therefore, it seems to be an optimal and convenient choice if patients can protect the teeth from acid by daily teeth brushing with a remineralization agent.

In recent years, many studies have demonstrated that bioglass ionic products are osteoproductive stimuli, demonstrating good impact on osteoblastic proliferation and differentiation, and consequently bone regeneration [8–10]. As a candidate for mineralization in dentistry, particularly

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in dentine remineralization, bioglass also plays an interesting role [11]. However, remineralization of enamel using bioglass has not been reported.

Our previous study showed that silicate-based bioceramics demonstrated good dental mineralization abilities [12]. Bioactive glasses are alkaline salts [13] and the bioactivity of the bioglasses was dependent on their chemical composition [14]. In aqueous solution, they are able to increase the pH value to reduce the enamel demineralization and proper compositions of bioactive glasses are even possible to result in a better teeth remineralization. Therefore, the aim of this study was to investigate the remineralization ability of bioglasses with different chemical compositions on acid-etched human dental enamel in saliva oral fluid (SOF) in vitro.

Materials and methods

The preparation for enamel specimens

Human dental enamel specimens with dentinoenamel junction (3 mm × 3 mm × 3 mm) were prepared by a water-cooled diamond saw. Then, specimens were ground with water-lubricated 600–1200 grit abrasive paper to obtain flat enamel surface. Subsequently, specimens were polished with diamond spray (1 μm, 0.5 μm) and polishing cloth, and then cleaned with ethanol solution in an ultrasonic bath for 3 min, rinsed under de-ionized water to remove smear layer and dried in ambient temperature.

Preparation of bioactive glass powders

Melt-derived 45S bioglass was prepared using high purity SiO_2, Na_2CO_3, $CaCO_3$, and P_2O_5 powders as raw materials. The powders were mixed and melted in a Pt crucible for 4 h at 1400 °C. Then the melt was quenched in water and ground in ethanol [15]. Sol–gel derived 58S and 77S bioglass powders were prepared by hydrolysis and polycondensation of tetraethyl orthosilicate (TEOS), triethyl phosphate (TEP), and $Ca(NO_3)_2{\cdot}4H_2O$, to get a sol. The gel was aged at 70 °C for 3 days and dried at 150 °C for 2 days, and then the obtained gel was heat treated for 2 h at 700 °C [16]. The structure and component of the bioglasses prepared were identified by X-ray diffraction analysis (XRD, Geigerflex, Rigaku, Japan) and X-ray fluorescence analysis (PW2404, Philips Co.). Glass powders were sieved to 300 mesh to obtain particles less than 50 μm in size.

Demineralization and remineralization of dental enamel

To simulate the demineralized enamel, enamel specimens were treated with 0.25% citric acid solution at pH 4.0 [17] and incubated at 37 °C for 10 min, then rinsed thoroughly with de-ionized water for further use.

Bioglass pastes were prepared with de-ionized water (L/P ratio of 1.0 mL g^{-1}). The surface of the enamel specimens were brushed with the pastes using a toothbrush for 3 min, and then rinsed with running water. This procedure was repeated three times. Then each treated specimen was placed in a polyethylene vial containing SOF in a total enamel surface area to liquid volume ratio of 1 cm^2/mL at 37 °C in a continuous shaking water bath [18]. The chemical composition of SOF is as follows: 8.38 mM KCl, 0.29 mM $MgCl_2{\cdot}6H_2O$, 1.13 mM $CaCl_2{\cdot}2H_2O$, 4.62 mM K_2HPO_4, 2.40 mM KH_2PO_4, and 0.022 ppm fluoride. The pH-value was adjusted to 7.2 using KOH and no precipitation was observed in the solution during the experimental period [19]. The SOF was refreshed every 24 h. After soaking for 7 days, the specimens were removed from the solution, rinsed thoroughly with de-ionized water and dried at room temperature.

Characterization of remineralized dental enamel

The structure as well as the morphology of the original, etched, and bioglass-treated enamel surface and cross section samples were analyzed by X-ray diffraction (XRD, Geigerflex, Rigaku, Japan) and observed by scanning electron microscopy (SEM, JSM-6700F, JEOL, Tokyo, Japan). The average calcium and phosphate molar ratio from three different locations on the surface of each sample was identified by energy dispersive X-ray spectroscopy (EDX, Oxford Instrument Co, UK.). The surface roughness of the original, etched, and bioglass-treated samples was measured using an atomic force microscope (AFM-Digital Instruments-Nanoscope III, Santa Barbara, CA, USA). Three scale parameters representing arithmetical average roughness (R_a), root mean square roughness (R_q), and the maximum peak to valley height (R_t) were determined [20]. Three different regions of each specimen in the size of 20 μm × 20 μm were measured and the values were expressed as mean standard deviation (SD) and analyzed using two-way analysis of variance (ANOVA). A p-value <0.05 was considered statistically significant.

Nanoindentation experiments using a TriboIndenter In Situ Nanomechanical Test System (Hysitron, Minneapolis, MN) were performed at maximum force of 1000 μN indentation load/unload with zero hold time, and loading rate was 200 μN/s. Each specimen was tested at ten random areas. Software associating with nanoindentation analyzed this data and calculated the nano-hardness and nano-reduced elastic modulus from the load–displacement curve recorded. Calculation was based on the power-law fitting method for unloading part of the load–displacement curve [21].

Results and discussions

Microstructure and components analysis of remineralized layer on the enamel surface

Three bioactive glasses, 45S, 58S, and 77S were obtained and their compositions and nominal compositions were listed in Tables 1 and 2. According to XRD analysis, their major structures are amorphous (Fig. 1). However, in 58S, some small-sized crystallites were revealed by XRD near 28 and 32 two theta in Fig. 1b. The XRD patterns of the original and etched enamel samples were shown in Fig. 2a and b as controls and those of the enamel surfaces treated by bioglasses and soaked in SOF for 7 days were shown in Fig. 2c, d, and e. The sharp, well-resolved Bragg peaks indicate the high degree of crystallinity of untreated enamel

Table 1 Bioactive glasses compositions (wt%)

Composition	SiO_2	CaO	Na_2O	P_2O_5
45S	44.8	26.5	23.4	5.3
58S	62.8	27.6	0	9.6
77S	73.7	16.5	0	9.8

Table 2 Bioactive glasses nominal compositions (wt%)

Composition	SiO_2	CaO	Na_2O	P_2O_5
45S	45	24.5	24.5	6
58S	60	36	0	4
77S	80	16	0	4

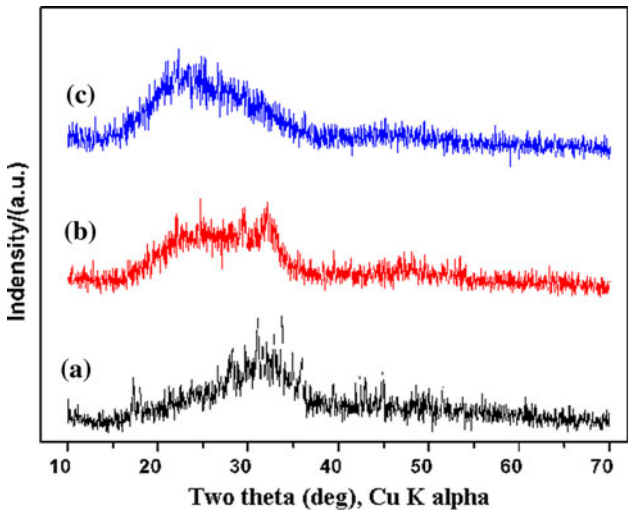

Fig. 1 XRD patterns of three different component bioactive glasses **a** 45S, **b** 58S, and **c** 77S

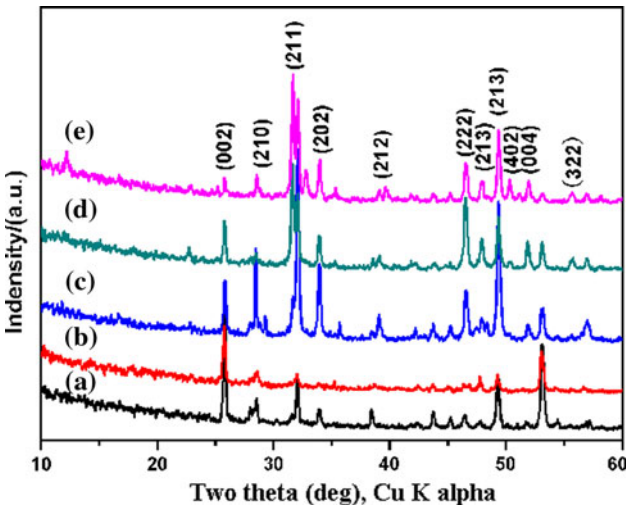

Fig. 2 XRD patterns of the human dental enamel surface after different treatment **a** original, **b** etched, **c** 45S bioactive glass treated, **d** 58S bioactive glass treated, and **e** 77S bioactive glass treated

(Fig. 2a). The only peaks recognized were those corresponding to the hydroxyapatite (HAp) phases, according to JCPDS card 09-0432. When the enamel was etched, its surface was softened and eventually demineralization process occurred with the dissolving of the HAp crystals starting from the enamel surface. Therefore, the decreasing of crystallinity was shown in XRD results [22, 23], the intensity of the characteristic peaks of HAp reducing and some peaks even disappearing (Fig. 2b). After the treatment with three bioactive glasses pastes and soaking in SOF for 7 days, new diffraction peaks appeared, suggesting new crystals formed on the enamel surfaces. The XRD patterns in Fig. 2c, d, and e confirmed that the newly formed crystals were apatite crystals. Furthermore, the major peaks in (210) and (202) for samples treated by 45S were the highest and those by 77S were the lowest.

The SEM micrographs of enamel samples are shown in Fig. 3. The polished original enamel surface was the smoothest and densest. When enamel surface was etched, HAp crystals on the enamel surface dissolved, leading to a relatively looser structure. When treatment with bioactive glasses pastes, the small micron-particles inserted and adhered in the grooves in the loosen enamel surface, after soaking in SOF for 7 days, apatite deposited on the enamel surface. SEM observation demonstrated that the roughness and the looseness of the enamel surfaces caused by etching were largely compensated by bioglasses treatment. 45S and 58S bioglasses showed similar ovoid crystals morphologies. The cross section of these two samples showed a thick mineralized layer and the average thickness was 4 and 2.5 μm, respectively. However, similar morphology was not found on 77S treated enamel surface at high magnification, only a thin, loose, and uneven layer of film was revealed by SEM in the cross-section image.

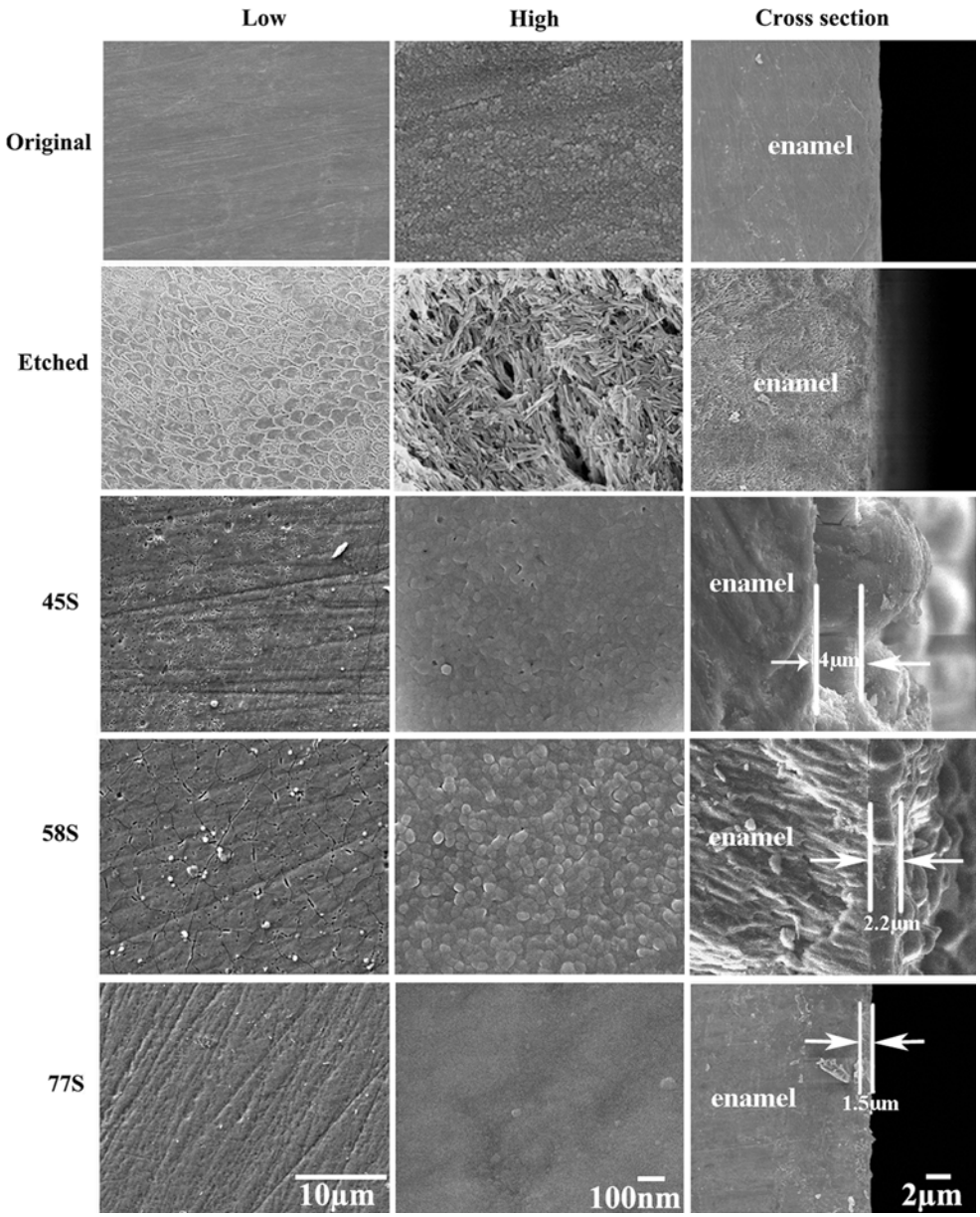

Fig. 3 SEM images of the surface (first and second columns) and cross section (last column) of the samples, including the original, etched, and treated with three bioactive glasses (45S, 58S, and 77S) then soaked in SOF for 7 days; Low and High indicated at low magnification and at high magnification, respectively; *Bar* size is the same in each group including low, high, and cross sections

Chemical composition is another important parameter to evaluate the mineralization ability of the bioglasses. The major components of the surfaces of all the treated samples were calcium and phosphorous determined by EDX and the average Ca/P molar ratios were showed in Table 3. It was clear that only 45S treatment yielded similar Ca/P molar ratio compared with the original enamel sample ($p > 0.05$), other treatments still had significant difference ($p < 0.05$). That is to say, 45S treatment yielded the closest chemical component to the natural enamel surface.

In fact, two important factors affect the remineralization of bioglasses. On one hand, since the preparation methods are different, the structure of melt-derived 45S was compacted and different to that of the sol–gel bioactive glass, which has a loose structure. Although 58S has higher silica content, it showed a similar mineralization activity as compared with the melt-derived 45S glass. One explanation is that, although the 45S glass contains sodium, which is much easier to release than the release of calcium ion from 58S, the surface reaction of the 58S glass with SOF is

Table 3 The average molar Ca/P ratio of enamel surface samples by EDX

Treated	Original	Etched	45S	58S	77S
Ca/P ratio	1.61 ± 0.02	1.50 ± 0.01*	1.60 ± 0.02	1.54 ± 0.02*	1.51 ± 0.01*

* Significant difference from the original samples, $p < 0.05$

faster because of the loose surface structure, and as a final result, both bioglasses showed similar mineralization ability. On the other hand, the sol–gel derived bioactive glasses such as 58S and 77S showed a composition dependent mineralization activity. When the glasses were soaked in SOF, network dissolution occurred by breaking of –Si–O–Si–O– bonds through the action of hydroxyl (OH) ions. Breaking of the network occurs locally which resulted in the release of silica into solution in the form of silicic acid [$Si(OH)_4$]. The rate of dissolution of silica depends very much on the glass composition. The dissolution rate decreases with the increase of SiO_2 because of the larger number of bridging oxygen bonds in the glass structure, at the same time, only little calcium ions released, leading to weak crystallization [24].

Surface roughness

Surface roughness of the teeth is an important aspect, as it may affect not only aesthetic properties but also bacteria adhesion and plaque formation in the oral microenvironment [25]. AFM was used to test the surface roughness of enamel specimens. The results were showed in Table 4. Etched enamel had a higher roughness value than the original one due to mineral-loss from the enamel surface. After treatment with each of the three bioactive glasses and soaking for 7 days, enamel surface became smooth in all cases, among which 77S treatment was the smoothest. This may due to the relatively less precipitation of the inorganic ions of SOF on the demineralized regions and crystalline phase formed was weak compared to the strong crystalline phase by 45S and 58S treatment. This was also proved by SEM observation and was described in the previous section. The improvement of the surface roughness by bioglass treatment was favorable for the resistance to bacteria adherence [26].

Table 5 Nano-hardness and nano-reduced elastic modulus of different enamel samples from Nanoindentation

Samples	Hv (GPa, SD)	Er (GPa, SD)
Original	4.27 ± 0.47	83.40 ± 7.1
Remineralized with 45S	5.37 ± 0.92*	108.88 ± 3.58*
Remineralized with 58S	4.49 ± 0.17*	117.88 ± 5.49*
Remineralized with 77S	1.18 ± 0.64*	51.47 ± 6.12*

* Significant difference from the original samples, $p < 0.05$

Mechanical properties

The mechanical properties of enamel samples are summarized in Table 5. In this experiment, polished enamel samples were used as a control instead of natural enamel. Polished and flattened enamel surface has generally a lower mechanical strength than natural enamel probably due to the removal of the upper prismatic surface, but the mechanical strength is dependent of different locations [27]. The mechanical properties of natural enamel have significant difference at different locations. Although there was a report by Jeng et al. [28] that nano-hardness and nano-reduced elastic modulus of the natural enamel surface were 5.29 ± 0.13 and 86.12 ± 1.01 GPa, respectively, they did not indicate the exact location they tested. There was another report that in the molars, from the cuspal regions to the enamel–dentine junction (EDJ), the hardness (Hv), and elastic modulus (Er) values are ranging from Hv < 3 GPa to Hv > 6 GPa, and Er < 70 GPa to Er > 115 GPa, respectively[29]. Therefore, the data from polished enamel samples was used for comparison. The results indicated that nano-hardness and nano-reduced elastic modulus of remineralized layer of 45S and 58S except 77S were significantly higher than that of the polished original samples ($p < 0.05$). Etched samples could not be measured due to the loose surface.

Table 4 Roughness parameters of enamel surface under different conditions from AFM

Samples	R_q (nm)	R_a (nm)	R_t (nm)	R_q/R_a
Original	82.36 ± 2.1	58.38 ± 3.7	976.94 ± 7.0	1.41
Acid etched	114.60 ± 4.4*	88.65 ± 3.6*	890.37 ± 5.4*	1.29
Remineralized with 45S	90.54 ± 0.72*	64.87 ± 1.48*	1056.0 ± 7.1*	1.40
Remineralized with 58S	142.73 ± 1.89*	76.60 ± 1.35*	1692.0 ± 5.78*	1.86
Remineralized with 77S	53.67 ± 1.11*	38.54 ± 0.74*	639.28 ± 4.84*	1.39

* Significant difference from the original samples, $p < 0.05$

In summary, the chemical composition of the bioactive glasses is critical to their remineralization ability. For 58S and 77S, both were made from the same sol–gel procedure, their mineralization ability is closely related to the silicon content. The 77S is richer in silicon content and lower in Ca/P ratio than 58S. On the contrary, 58S bioglass, thanks to its higher calcium ions, its stability of surface carbonates is higher [24], and its mineralization effect is stronger. Although 45S and 58S bioglasses had relatively better mineralization ability, since 45S contains sodium, which is not in the component of 58S, the existence of sodium affected the dissolving rate of calcium ion in 45S. The releasing of sodium ion would accelerate dissolution of calcium ion, ultimately affecting the HAp formation roughness and mechanical properties of the treated enamel surface, leaving 45S the best among the three bioglasses tested. In addition, since microstructure of the mineralized layer with 45S and 58S bioglasses, including HAp crystallites arrangement and remineralization potential of the bioglasses themselves, both affected their mechanical properties, the bonding strength between the mineralized layer and the enamel matrix needs to be further discussed to reduce the risk of the removal of the precipitate layers by daily brushing.

Conclusions

Definite compositions of silicate-based bioactive glasses have been effectively used as remineralized agents, such as 45S and 58S, to repair the acid-etched dental enamel. Compared to 58S, 45S treated enamel surface had more compact and smoother mineralized layer and the mechanical strength and surface roughness was significantly improved. Therefore, a proper composition of silicate-based bioactive glass can improve the teeth remineralization ability and may be a potential candidate as a toothpaste agent.

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