Acidity-induced release of zinc ion from BioUnion™ filler and its inhibitory effects against Streptococcus mutans

Yuhan LIU¹, Tomoki KOHNO², Ririko TSUBOI², Haruaki KITAGAWA¹ and Satoshi IMAZATO¹,²

INTRODUCTION

BioUnion filler incorporated into restorative/coating materials is a new bio-functional glass powder. The most unique function of BioUnion filler is its ability to release Zn²⁺ in acidic environments. In this study, the ion release profile of BioUnion filler under acidic conditions and its antibacterial effects against Streptococcus mutans were evaluated. The concentrations of Zn²⁺ released from BioUnion fillers into acetic acids were greater than those released into water. S. mutans inhibition by BioUnion fillers was greater with sucrose than without sucrose, reflecting a decrease in suspension pH in response to the addition of sucrose. Exposure to acids increased Zn²⁺ release from BioUnion fillers, and the fillers after repeated exposure to acids demonstrated inhibitory effects against S. mutans. These findings suggest that BioUnion filler accelerated the release of Zn²⁺ under acidic conditions, which induced bactericidal/inhibitory effects against S. mutans.

Keywords: BioUnion filler, Zinc, Restorative material, Antibacterial effect
for 24 h with shaking at 100 rpm, 200 µL of eluate was collected and the concentration of ions was determined. The eluates were diluted in 4.8 mL distilled water, after which the concentrations of Zn$^{2+}$ and Ca$^{2+}$ were measured using an inductively coupled plasma-optical emission spectrometer (ICP-OES; iCAP 7000 Series, Thermo Scientific, Cambridge, UK). The concentration of F$^{-}$ was determined using a fluoride ion electrode (FIE; 6561S-10C, HORIBA, Kyoto, Japan). The experiments were repeated four times.

**X-ray diffraction (XRD) analysis of BioUnion filler after immersion in water and acid**

A total of 200 mg of BU was immersed in 10 mL of water (pH 7.0), acetic acid (pH 4.5), or hydrochloric acid (pH 1.2). After storage at 37°C for 24 h with gyratory shaking at 100 rpm, the particles were dried and mounted on an XRD apparatus (RINT-Ultima2100, Rigaku, Tokyo, Japan). The X-ray beam angle 2$\theta$ (degrees) range was set between 10 and 75 degrees and scanned at 0.02 degrees per second. The Cu X-ray source was operated with an acceleration voltage of 40 kV and an electron beam current of 30 mA. Peak positions in the XRD patterns obtained from the specimens were compared and matched with those of the standard material in the powder diffraction file of the International Center for Diffraction Data 2013.

**Measurement of minimum inhibitory concentrations (MICs) of Zn$^{2+}$, Ca$^{2+}$, and F against S. mutans**

To evaluate the concentrations of Zn$^{2+}$, Ca$^{2+}$, and F$^{-}$ that effectively inhibited S. mutans growth, MICs of these ions against S. mutans were measured. ZnCl$_2$, CaCl$_2$ (Wako, Osaka, Japan), and NaF (Sigma-Aldrich, Tokyo, Japan) were dissolved in distilled water to obtain standard solutions of Zn$^{2+}$, Ca$^{2+}$, and F$^{-}$, respectively. The MIC values of Zn$^{2+}$, Ca$^{2+}$, and F$^{-}$ against S. mutans NCTC 10449 were measured using a microdilution assay. Briefly, 50 µL aliquots of each standard solution at 0.5–2,048 ppm with serial two-fold dilutions were prepared in the wells of a 96-well microplate. Next, 50 µL of S. mutans suspension in Brain Heart Infusion (BHI) broth (Becton Dickinson, Sparks, MD, USA) from the stock culture were incubated for 12 h, after which the sample was adjusted to 2.0×10$^6$ colony-forming units (CFU)/mL and added to the well, resulting in a four-fold dilution of the original solution. The microplates were subsequently incubated anaerobically at 37°C for 24 h, after which the MIC value was determined as the lowest concentration at which turbidity was not observed by visual examination. The experiments were repeated five times.

**Evaluation of antibacterial activities of BioUnion filler**

The S. mutans suspension was adjusted to approximately 1.0×10$^4$ CFU/mL in BHI broth, after which 40 mg of BUs were placed in a well of a 96-well microplate and immersed in 200 µL of S. mutans suspension (approximately 2.0×10$^5$ CFU) supplemented with or without 1% sucrose (Wako). FGs were used as a control. After anaerobic incubation at 37°C for 24 h with gyratory shaking at 100 rpm, 100 µL of S. mutans suspension was collected and added into 9.9 mL of BHI broth. The suspension was then serially diluted with BHI broth and inoculated on BHI agar (Becton Dickinson) plates. The plates were incubated anaerobically at 37°C for 24 h, after which the number of colonies formed was determined. All experiments were repeated three times.

**Evaluation of Zn$^{2+}$ release from BioUnion filler with repeated exposure to acids and inhibition of S. mutans**

A total of 40 mg of BU were placed in one transwell insert, then immersed in 300 µL of acetic acid (pH 4.5 or 5.5) at 37°C for 1 day. Next, the particles were immersed in distilled water (pH 7.0) and stored for 3 days at 37°C while replacing the water every day. This procedure was repeated three times until Day 10 (i.e., the particles were immersed in acetic acid on Days 0–1, 4–5 and 8–9 and in distilled water on Days 1–4, 5–8, and 9–10). The eluates were collected on Days 1–10 and diluted in 5 mL distilled water. The concentrations of Zn$^{2+}$ were measured using ICP-OES as described above. All experiments were repeated five times.

To evaluate the antibacterial effects, particles were collected on Day 8 (after two exposures to acetic acid at pH 4.5) using the same methods as described above. The S. mutans suspension was adjusted to approximately 1.0×10$^4$ CFU/mL in BHI broth with 1% sucrose. On Day 8, the particles were transferred to a well of a 96-well microplate and incubated with 200 µL of S. mutans suspension (approximately 2.0×10$^5$ CFU). After anaerobic incubation for 24 h with gyratory shaking at 100 rpm, the viable bacteria were counted. All experiments were repeated three times.

**Statistical analysis**

Statistical analyses were performed using SPSS Statistics 21 (IBM, Chicago, IL, USA). The homogeneity of variances was initially confirmed. The results for ion release and bacterial growth were statistically analyzed by analysis of variance (ANOVA) and Tukey’s honestly significant difference (HSD) test. A p<0.05 was considered to indicate significance.
RESULTS

Ion release from BioUnion filler in different solutions
The concentrations of Zn$^{2+}$, Ca$^{2+}$, and F$^-$ released from BU and FG into distilled water and acetic acid solutions are shown in Table 1. The concentrations of Zn$^{2+}$ and Ca$^{2+}$ released from BU into acetic acid at pH 4.5 were approximately 85 or 73 times higher than those released into water (p<0.05, ANOVA, Tukey’s HSD test). The concentrations of Zn$^{2+}$ and Ca$^{2+}$ released into acetic acid at pH 5.5 were significantly lower than those released into acetic acid at pH 4.5, but higher than those released into water. Conversely, the concentrations of F$^-$ released into acetic acids at both pH 4.5 and 5.5 were significantly lower than those released into water.

Zn$^{2+}$ and Ca$^{2+}$ released from FG was not found in all eluates and the concentrations of F$^-$ released from FG into acetic acid solutions at pH 4.5 and 5.5 were significantly lower than those released into water.

XRD analysis of BioUnion fillers after immersion in water and acids
The XRD patterns of BU after immersion in water, acetic acid, and hydrochloric acid are shown in Fig. 2. Insoluble lanthanum fluoride (LaF$_3$) was detected on BU after immersion in acetic and hydrochloric acids.

MICs of Zn$^{2+}$, Ca$^{2+}$, and F$^-$
The MIC values of Zn$^{2+}$, Ca$^{2+}$, and F$^-$ against S. mutans NCTC10449 were determined to be 64, >512, and 128 ppm, respectively.

Antibacterial activities of BioUnion filler
Figure 3 shows the number of viable bacteria after incubation in the presence of BU and FG. After 24 h of incubation without the addition of sucrose, colony counts of viable S. mutans in the presence of FG were significantly lower than in the control without any particles. The number of surviving cells in the presence of BU (approximately 1.3×10$^5$ CFU) was further decreased compared with FG (p<0.05, ANOVA, Tukey’s HSD test).

After being cultured with sucrose, the number of viable cells in the presence of BU (approximately 4.4×10$^2$ CFU) was lower than the initial amount of bacteria (approximately 2.0×10$^3$ CFU) used for this experiment,
indicating that BU exhibited a bactericidal effect against S. mutans. In addition, its inhibition of S. mutans by BU was greater for the culture with sucrose than without sucrose (p<0.05, ANOVA, Tukey’s HSD test).

**Release of Zn^{2+} from BioUnion filler with repeated exposure to acids and inhibition of S. mutans**

Release profiles of Zn^{2+} from BU with repeated exposure to acetic acids at pH 4.5 or 5.5 are shown in Fig. 4A. On Day 1, 505.5±12.6 ppm of Zn^{2+} was released into acetic acid at pH 4.5. The concentrations of Zn^{2+} in water on Days 2–4 decreased dramatically to 29.6±10.1 ppm. When the particles were again immersed in acetic acid at pH 4.5, the amount of Zn^{2+} released on Days 5 and 9 increased to 418.8±1.8 and 349.8±9.3 ppm, respectively. The concentrations released into acetic acid (pH 5.5) on Days 1, 5 and 9 were much lower than those released into acetic acid (pH 4.5) at each time-point (p<0.05, Student’s t-test). Although the concentrations of Zn^{2+} released into both acetic acids on Days 5 and 9 decreased compared with those released on Day 1, the concentrations of Zn^{2+} released from BU increased repeatedly in response to exposure to acetic acids. Additionally, the concentrations released into both acetic acids at pH 4.5 and 5.5 were above the MIC value of Zn^{2+} against S. mutans.

Figure 4B shows the number of viable bacteria in the presence of BU and FG, which were collected on Day 8 after two exposures to acetic acid at pH 4.5. BU significantly inhibited the growth of S. mutans compared with the control and FG (p<0.05, ANOVA, Tukey’s HSD test).

**DISCUSSION**

Hench et al. developed a bioactive glass composed of SiO₂, Na₂O, CaO, and P₂O₅. This bioactive glass is a potential candidate for use as filler particles in restorative materials, because it can enhance hard tissue regeneration and exert some antimicrobial effects by releasing ions. Its antimicrobial effects are attributed to the release of ions such as Ca²⁺, which causes neutralization of the local acidic environment and leads to a local increase in pH that is not well tolerated by bacteria. Fluoride-containing bioactive glass that can release and recharge fluoride has been developed to provide cariostatic effects. Fluoride-containing bioactive glass that can release and recharge fluoride has been developed to provide cariostatic effects. The inhibitory mechanisms of F⁻ against S. mutans are known to be related to their metabolic activity. Specifically, F⁻ inhibits enolase, which catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the Embden-Meyerhof-Parnas...
pathway during bacterial glycolytic metabolism. Fluoride at high concentrations is known to exert antibacterial activity against oral bacteria. The minimal bactericidal concentration (MBC) of NaF against S. mutans UA159 is reportedly 2,500 µg/mL\(^{30}\). Such high concentrations of F\(^-\) are difficult to release from bioactive glasses; therefore, the antimicrobial effects of fluoride-containing bioactive glass are limited. As a result, additional components are needed to more effectively demonstrate antibacterial effects against oral microorganisms.

BioUnion filler is a silicon-based glass structure containing zinc, calcium, and fluoride. Zinc or zinc compounds are known to exhibit antibacterial effects against oral bacteria. The MIC/MBC values of zinc against S. mutans were reported to be lower than those of fluoride\(^{17,19,31}\). The results obtained in this study also confirmed that the MIC value of Zn\(^{2+}\) was 64 ppm, which was lower than that of F\(^-\) at 128 ppm.

Because BioUnion filler can be dissolved in acids via an acid-base reaction, Zn\(^{2+}\), Ca\(^{2+}\), and F\(^-\) in glass may be released under acidic conditions. To evaluate use of this unique function to promote ion release under acidic conditions, profiles of Zn\(^{2+}\), Ca\(^{2+}\), and F\(^-\) release from BioUnion filler into acids were evaluated. A powder of Fuji VII, which is capable of releasing high concentration of F\(^-\)\(^{32-34}\), was used as a control. To determine the pH values of the acids used for release tests, pH changes during incubation of acidogenic S. mutans NCTC10449 were confirmed by measuring the pH values of the suspensions. After incubation for 24 h, the pH values of S. mutans suspensions with and without sucrose gradually decreased to 4.3 and 5.4, respectively, where they were maintained (Fig. 5). Based on these results, acetic acid solutions at pH 4.5 and 5.5 were used for release tests. The concentrations of Zn\(^{2+}\) and Ca\(^{2+}\) released into both acetic acid solutions (pH 4.5 and 5.5) were higher than those released into water (Table). In addition, the concentrations of Zn\(^{2+}\) released into both acids (pH 4.5 and 5.5) were higher than the MIC value of Zn\(^{2+}\) against S. mutans. Conversely, the concentration of Zn\(^{2+}\) released into water was less than the MIC value of Zn\(^{2+}\) against S. mutans. The concentrations of Ca\(^{2+}\) released into both water and acetic acids were lower than the MIC values of Ca\(^{2+}\) against S. mutans. These results indicated that the releases of cationic Zn\(^{2+}\) and Ca\(^{2+}\) from BioUnion fillers were accelerated under acidic conditions (Fig. 6), and that Zn\(^{2+}\) released from BioUnion fillers would effectively inhibit S. mutans growth.

For F\(^-\), concentrations released from BioUnion fillers into acetic acid were lower than those released into water. XRD analysis (Fig. 2) indicated that LaF\(_3\) was detected on BioUnion fillers after immersion in acids. No specific peaks derived from precipitates of Fuji VII powders after immersion in acetic acid were observed in the XRD patterns (data not shown). It is considered that F\(^-\) released from BioUnion fillers in acids reacted with La\(^{3+}\) (probably released from the fillers) and formed insoluble LaF\(_3\), thereby lowering the concentration of F\(^-\) released from BioUnion fillers that was detected in acids.

To evaluate the antibacterial effects of BioUnion filler and Fuji VII powder, S. mutans suspensions with or without added sucrose were incubated in the presence of these powders. The results confirmed that the release of Zn\(^{2+}\) from BioUnion fillers more effectively inhibited S. mutans than that released from Fuji VII powder. Moreover, BioUnion fillers demonstrated bactericidal effects against S. mutans when sucrose was added,
whereas they exhibited inhibitory effects against \textit{S. mutans} growth in the absence of sucrose. As described above, the pH value of \textit{S. mutans} suspensions with added sucrose was lower after 24 h of incubation than that of suspensions without added sucrose. Therefore, the inhibition of \textit{S. mutans} by BioUnion filler was greater for the culture with sucrose than without sucrose, reflecting the decrease in the pH of the suspension in response to the addition of sucrose. We measured the MBC of Zn\textsuperscript{2+} against \textit{S. mutans} NCTC10449 and found that it was >512 ppm (data not shown). While the concentrations of Zn\textsuperscript{2+} released into both acetic acids at pH 4.5 and 5.5 were lower than the MBC values, higher concentrations of Zn\textsuperscript{2+} were released in response to the incubation of \textit{S. mutans} with sucrose than without sucrose, resulting in bactericidal effects against \textit{S. mutans}. Although the detailed mechanism underlying the antibacterial effects of Zn\textsuperscript{2+} has not been fully elucidated, it has been reported that Zn\textsuperscript{2+} prevented the synthesis of bacterial cell walls\textsuperscript{17}. Other studies have suggested that zinc acts directly by altering cell proteins \textit{via} processes such as transmembrane proton translocation, or indirectly by inhibiting protease induced bacterial adhesion\textsuperscript{35,36}. Based on these antibacterial mechanisms of Zn\textsuperscript{2+}, the acidity-induced release of Zn\textsuperscript{2+} from BioUnion fillers exhibited bactericidal or inhibitory effects against \textit{S. mutans}.

Once dental plaque is formed on the surfaces of teeth or materials, the pH values around them are decreased by acids produced from oral bacteria such as \textit{S. mutans}\textsuperscript{37}. When these acidogenic bacteria produce acids, BioUnion filler incorporated into materials is capable of effectively releasing Zn\textsuperscript{2+}. Therefore, we confirmed the ability of BioUnion fillers to release Zn\textsuperscript{2+} with repeated exposure to acids and the inhibitory effects against \textit{S. mutans}. When the particles were immersed in acetic acids (pH 4.5 and 5.5), the concentrations of Zn\textsuperscript{2+} from BioUnion fillers increased (Fig. 4A). During three exposures to acetic acids at pH 4.5 and 5.5, the effective concentrations above the MIC values of Zn\textsuperscript{2+} against \textit{S. mutans} could be maintained. Moreover, the particles after two rounds of exposure to acetic acid (pH 4.5) still inhibited the growth of \textit{S. mutans} in the presence of sucrose (Fig. 4B). However, the concentrations of Zn\textsuperscript{2+} released into the acids gradually decreased after two or three exposures. It has been reported that ions that act as network modifiers in the structures of bioactive glass such as 45S5 can be released under acidic conditions by dissolution of the surfaces of particles\textsuperscript{38,39}. Moreover, the release of Zn\textsuperscript{2+} in acids is believed to be accelerated by dissolution of the particles, but to gradually decrease after repeated exposure to acid with decreasing surface area of the BioUnion filler. Therefore, the Day 8 samples, which had been exposed to acidic solutions twice, did not exhibit bactericidal effects against \textit{S. mutans} and instead exerted only inhibitory effects. To maintain the bactericidal effects of Zn\textsuperscript{2+} released, the ability of BioUnion filler itself or materials containing BioUnion fillers to be maintained at the level needed to kill \textit{S. mutans} by recharging with zinc ion solution is needed.

Dental plaque includes multiple microbial species. Among them, \textit{S. mutans} is one of the most representative acidogenic and cariogenic bacteria. In this study, the antibacterial effects of BioUnion fillers on \textit{S. mutans} growth was evaluated \textit{in vitro}. However, further studies to evaluate its antibacterial effects against other bacteria are needed. In addition, because saliva continuously flows in the oral cavity, the ability of BioUnion fillers and materials incorporating these particles to release Zn\textsuperscript{2+} and inhibit bacteria or biofilms should be evaluated in a clinically-relevant setting.

**CONCLUSION**

A new bio-functional glass powder, BioUnion filler, incorporated in commercial restorative/coating materials was capable of releasing Zn\textsuperscript{2+}, Ca\textsuperscript{2+}, and F\textsuperscript{−}. Under acidic conditions, BioUnion filler demonstrated accelerated release of Zn\textsuperscript{2+}, which exerted bactericidal or inhibitory effects against \textit{S. mutans}. Such acidity-induced release of Zn\textsuperscript{2+} has the potential to hinder plaque formation on the surfaces of materials incorporating BioUnion filler.

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