A Chemical Approach to Optimizing Bioactive Glass Dental Composites

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Abstract

The chemical microenvironment surrounding dental composites plays a crucial role in controlling the bacteria grown on these specialized surfaces. In this study, we report a scanning electrochemical microscopy (SECM)–based analytic technique to design and optimize metal ion-releasing bioactive glass (BAG) composites, which showed a significant reduction in biofilm growth. SECM allows positioning of the probe without touching the substrate while mapping the chemical parameters in 3-dimensional space above the substrate. Using SECM and a solid-state H^+ and Ca^{2+} ion-selective microprobe, we determined that the local Ca^{2+} concentration released by different composites was 10 to 224 μM for a BAG particle size of <5 to 150 μm in the presence of artificial saliva at pH 4.5. The local pH was constant above the composites in the same saliva solution. The released amount of Ca^{2+} was determined to be maximal for particles <38 μm and a BAG volume fraction of 0.32. This optimized BAG-resin composite also showed significant inhibition of biofilm growth ($24 \pm 5 \mu m$) in comparison with resin-only composites ($53 \pm 6 \mu m$) after *Streptococcus mutans* bacteria were grown for 3 d in a basal medium mucin solution. Biofilm morphology and its subsequent volume, as determined by the SECM imaging technique, was $(0.59 \pm 0.38) \times 10^7 \mu m^3$ for BAG-resin composites and $(1.29 \pm 0.53) \times 10^7 \mu m^3$ for resin-only composites. This study thus lays the foundation for a new analytic technique for designing dental composites that are based on the chemical microenvironment created by biomaterials to which bacteria have been exposed.

Keywords: biofilm, scanning electrochemical microscopy, local pH, calcium ions, oral biofilm, antimicrobial surface

Introduction

The main reason that dental composite restorations are replaced is due to a recurrence of caries in the vicinity of the existing restoration, often at the margin with the tooth structure (Mjör 1998; Demarco et al. 2012; Opdam et al. 2014; Ástvaldsdóttir et al. 2015). This process is believed to be caused by the production of acid from the bacterial biofilm that forms on the tooth and composite surface, leading to demineralization and potential degradation of the composite and adhesive interface. Research into the development of dental composite and adhesive formulations possibly containing antimicrobial- and anti-biofilm-forming agents is a direct response to this problem (Koo et al. 2017).

Bioactive glass (BAG) is a unique material that was recently suggested for a variety of applications in medicine and dentistry (Jones 2013), including stimulating bone formation (Jia et al. 2018), desensitizing teeth (Wang et al. 2010; Mitchell et al. 2011; Lopez et al. 2017), remineralizing white spot lesions (Bakry et al. 2018), and restoring teeth (Par et al. 2018). BAG was also recently suggested as an additive to resin-based dental composites for the purpose of releasing metal ions and influencing the formation of oral biofilms (Xu et al. 2015; Khvostenko et al. 2016). Most BAG-containing composites have been characterized for their mechanical properties and for their ion release (Ca^{2+}) into solution by spectroscopic measurements (inductively coupled plasma–mass spectrometry,

inductively coupled plasma–atomic emission spectrometry, or atomic absorption). These chemical characterizations typically involve collecting an aliquot of the sample solution and measuring the amount of Ca^{2+} without indicating the concentration of Ca^{2+} released in real time or the concentration to which the bacteria are exposed while growing on the composite surface. Both these phenomena are critical to understanding how to formulate bioactive composites that are capable of disrupting biofilm formation. Our earlier scanning electrochemical microscopy (SECM)–based studies showed that pure BAG specimens were able to shift the local pH from 4.5 to 6.2 while maintaining the local Ca^{2+} concentration at 1.2 mM in artificial saliva (Ummadi et al. 2016). SECM is a surface probe technique (Amemiya et al. 2008) that allows precise (within 10 μm) positioning of the electrochemical probe over the substrate of interest (e.g., biofilm, composite) without touching or destroying it, thus making it a real-time noninvasive method. The electrochemical

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probe is positioned by using an amperometric approach curve that correlates the current probe record with the distance between the probe and substrate. In general, the approach curve is performed in the presence of a redox molecule that can be removed after positioning the probe at a known distance from the substrate.

In this study, we used our newly developed SECM-based electroanalytic technique to chemically characterize BAG-containing composites to quantify the local Ca^{2+} concentration to further develop optimal BAG-containing resin composites. The hypothesis to be tested was that a dental composite containing a specific size and volume fraction of BAG could be formulated to produce a uniform local concentration of Ca^{2+} above its surface and thereby disrupt biofilm formation or mitigate its effects.

Materials and Methods

Materials

For details about materials, see the Appendix.

Instrumentation

All electrochemical measurements were performed with a CHI bipotentiostat (CH Instruments) and a scanning electrochemical microscope (model 920D; CH Instruments). Ag/AgCl and a 0.5-mm Pt wire were used as reference and counter electrodes, respectively.

Bacterial Strains and Growth Conditions

Streptococcus mutans (*S. mutans*) was cultured on brain heart infusion agar plates at 37 °C in 5% CO_2 environments (Pakula and Walczak 1963; Arweiler and Netuschil 2016). Prior to all biofilm-mapping experiments, *S. mutans* was grown for 12 h in brain heart infusion media.

Fabrication of pH and Ca^{2+} Ion-Selective Microelectrode Sensors for the SECM Chemical Probe

Details about the fabrication and electrochemical characterization of the pH and Ca^{2+} ion-selective electrodes were previously described (Ummadi et al. 2016; Joshi et al. 2017). In brief, a 1-mm-diameter borosilicate glass capillary was pulled with a gravity puller to make a 25- to 35- μm -diameter pipette. A sensing membrane containing the following ion-selective cocktail was then packed through the back end of the pipette: 5% proton ionophore-I or ETH-129 (Ca^{2+} ionophore), 2% potassium tetrakis (4-chlorophenyl) borate, 30% NPOE, 3% PVC, and 60% Vulcan carbon powder. Both the pH and the Ca^{2+} ion-selective microelectrode sensors were calibrated in artificial saliva at 23 °C.

Fabrication of BAG-Resin Composites

Bioactive Glass. BAG that contained oxides of Si (65%), Ca (31%), and P (4%; mole percentage) was prepared as previously described (Davis et al. 2014). Following the heating regimen, BAG was obtained as small transparent colorless pieces that were ball milled (ceramic vessel and balls) in ethanol to yield a slurry of fine powder. After evaporation of the ethanol at 37 °C, the powder was passed through a series of sieves (150, 125, 90, 75, 45, and 38 μm) to yield 5 particle-sized distribution groups: 150 to 125 μm , 90 to 75 μm , 45 to 38 μm , <38 μm , and $\leq 5 \mu\text{m}$. The fifth group ($\leq 5 \mu\text{m}$) was obtained by micronizing (Sturtevant Inc.) the ball-milled powder. For details, see the Preparation of Bioactive Glass section in the Appendix.

Composites. Composites were prepared by mixing a resin composed of 50 wt% triethyleneglycoldimethacrylate (Esstech Inc.) and 50 wt% bis-phenol-A-glycidyl methacrylate (Esstech Inc.) with BAG powder in a centrifugal mixing system operating at 1,900, 2,400, and 2,900 rpm 1 min each (DAC-150 Speedmixer; Flaktek Inc.). The resin also contained a photoinitiator, camphorquinone (0.4%: Sigma Aldrich Inc.); a co-initiator, ethyl-p-dimethylaminobenzoate (0.8%: Esstech Inc.); and an inhibitor, butylated hydroxytoluene (0.05%: Acros Organics). The composite formulations were based on volume fractions (V_f) of BAG (density = 2.32 g mL^{-1}) and resin (density = 1.16 g mL^{-1}). Composites with 0.32 V_f (48.5 wt%) of BAG were prepared for each of the 5 BAG particle-sized groups, yielding 5 composite formulations with estimated interparticle spacing, D_s , of 4.3, 42.5, 58.8, 116.9, and 187.7 μm (Table and Appendix Table). D_s was estimated with the following formula, originally proposed for spherical particles (Ferracane et al. 1986):

$$D_s = (2 \cdot d_p)(1 - V_f) / 3 \cdot V_f,$$

where d_p is the average particle size and V_f is the filler volume fraction.

Specimen Preparations. Resin composite was loaded into a disk-shaped mold (5 × 2 mm) between glass slides and irradiated (7-mm diameter light tip, 800 mW cm^{-2} ; Demi, KaVo Kerr) for 40 s per side ($n = 5$). The cured disks were lightly abraded (4,000 grit paper) on each surface, and the sides covered with nail polish.

Characterization of the BAG-Containing Resin Composites with SECM

A pH and Ca^{2+} ion-selective microelectrode was used as a chemical probe in SECM to determine the local concentration of Ca^{2+} released above BAG-containing composites (20 μm above the surface) and the corresponding change in local pH in the presence of artificial saliva (pH 4.5). The pH and Ca^{2+} ion-selective microelectrodes were used individually to measure

Table. Morphology Data Obtained from the Biofilm Grown on the Optimized BAG-Resin Composite and Its Controls.

	Estimated Average d_p , μm	D_s , μm	Biofilm Volume, $\mu\text{m}^3 \times 10^7$	Average Biofilm Height, μm
Glass substrates	—	—	1.41 ± 0.29	60.04 ± 5.04
Resin substrates	—	—	1.29 ± 0.53	53.34 ± 6.46
BAG particle composites, μm				
<5	3	4.3	0.59 ± 0.38	24.33 ± 4.78
<38	30	42.5	0.52 ± 0.052	22.73 ± 3.54
38 to 45	41.5	58.8	0.64 ± 0.13	27.92 ± 3.22
75 to 90	82.5	116.9	0.67 ± 0.055	28.46 ± 3.66
125 to 150	132.5	187.7	0.81 ± 0.19	35.08 ± 4.53

Each trial was performed 3 times with 2 samples of each resin per trial. BAG, bioactive glass; d_p , particle diameter; D_s , interparticle distance.

Figure 1. Chemical characterization of bioactive glass composites. (A) Experimental setup for calcium ion mapping above the bioactive glass-containing resin with scanning electrochemical microscopy. (B) Points on the resin at which the z-scans were taken. Representative z-directional (C) Calcium scans and (D) pH scans from 20 to 1,000 μm above the resin surface during exposure to artificial saliva at pH 4.5.

the corresponding parameters in separate experiments. At first, the microsensor was positioned by using an amperometric approach curve (Ummadi et al. 2016) in the presence of 1 mM ferrocyanide in artificial saliva (pH 7.2). Later, the solution was replaced with artificial saliva at pH 4.5, and the sensor was turned on to measure potential versus time. All the measurements were taken at steady state (approximately after 1 min). For z-scan experiments, the probe was moved from 20 to 1,000 μm above the surface at different spots over an area of $1,000 \times 1,000 \mu\text{m}$ (Fig. 1A, B) while continuously measuring the potential. All potentials were converted to concentrations with a calibration curve. All results are reported as changes in Ca^{2+} concentration relative to artificial saliva ($700 \mu\text{M} \text{Ca}^{2+}$).

Growing *S. mutans* Biofilm on BAG-Containing Resin Composites

S. mutans, the major oral acid-producing bacteria (Hamada and Slade 1980; Dibdin and Shellis 1988; Guo et al. 2013; Krzyściak

et al. 2013) were grown on different BAG composites to test the efficacy of these substrates in controlling the growth of biofilm. Unfilled resin specimens, without BAG particles, were used as control substrates. Two resin specimens and two BAG-containing composite disks were used for each biofilm experiment. The disks were polished with 400/P800 grit sandpaper, covered with Kapton tape such that only a 2-mm-diameter circle of the substrate was exposed, and placed in a 30-mm petri dish on double-sided tape. Bacteria (optical density: 0.1) were then inoculated in the dish for 2 h in the presence of 150 mM sucrose in basal medium mucin (BMM) solution, followed by flowing BMM (3.6 mL/h) through the dish at 37 °C for 72 h. BMM flow was paused every 8 h, and 150mM sucrose and BMM solution were added to the dish for 15 min. A separate syringe was used as a sterile method to add the intermittent sucrose. Airflow of 2 L/min was maintained throughout the experiment to ensure a constant positive pressure environment. At the end of the 72 h, the petri dish was transferred to the biosafety cabinet; the Kapton tape was carefully removed from the sample; and the BAG-biofilm specimen was placed on the SECM stage for further experiments (Fig. 2).

Determination of Volume and Morphology of *S. mutans* Biofilm Grown on Composites by SECM

S. mutans biofilms were grown as described in the previous section. The biofilm-containing composite samples were fixed inside the petri dish with double-sided tape and placed on the SECM stage (Fig. 2). A 2 mM solution of ferrocyanide (non-toxic redox molecule) in artificial saliva at pH 7.2 was added to the dish. To determine biofilm height and morphology, a 25- μm -diameter Pt electrode (redox sensor) was used in this experiment. Ag/AgCl was used as the reference electrode, and stainless-steel wire was used as the counter electrode. A probe approach curve (Harris et al. 2016) was then performed on the composite and on the biofilm to determine its height (Appendix Fig.). The probe approach curve allowed the positioning of the SECM probe at a precise distance above the surface without touching or destroying the sample. A series of probe approach curves were performed on the biofilm to determine the average biofilm height across the entire 2-mm-diameter biofilm substrate, thus allowing a calculation of biofilm volume. Later, live biofilm morphologic imaging was performed with SECM

to determine the biofilm volume. This imaging was carried out at a distance of 17.5 μm above the biofilm at a speed of 50 $\mu\text{m/s}$ (2,500 pixels). To obtain a more representative profile of the biofilm, each substrate type was imaged 18 times over the course of 3 separate trials. The redox probe or 25- μm -diameter Pt electrode was biased at +0.4 V during the probe approach curve and constant-distance SECM imaging. A paired *t* test was used to compare the biofilm growth on different substrates ($\alpha = 0.05$).

Results

Quantification of the Local Chemical Environment above BAG-Containing Resin Composites

The *z*-directional Ca^{2+} profile of BAG-resin composites with particle sizes ranging from <5 μm to 150 μm extended to 600 to 800 μm above the composite surface (Fig. 1C). The *z*-directional pH profile showed a subtle elevation in pH (i.e., a change of only 0.05 to 0.3 pH units) directly at the surface for the same formulations, with the effect decreasing to pH 4.5 within 600 to 800 μm above the composite surface (Fig. 1D).

The change in local concentration very near the surface (at *z* = 20 μm) was 100, 88, 166, 213, and 225 μM for BAG particle sizes of 125 to 150, 75 to 90, 38 to 45, <38, and <5 μm , respectively, relative to artificial saliva that had a starting Ca^{2+} concentration of 700 μM at pH 4.5 (Fig. 3A). The pH changes for these formulations at *z* = 20 μm ranged from 0.05 to 0.2 units.

Measuring Biofilm Morphology on BAG-resin Composites

The mean $\pm$ SD height of biofilms grown on the BAG-containing resin substrate with a particle size <5 μm and $V_f = 0.32$ was 24 ± 5 μm , with a corresponding estimated volume of $(0.59 \pm 0.38) \times 10^7$ μm^3 ; for a particle size <38 μm , 23 ± 4 μm and $(0.52 \pm 0.052) \times 10^7$ μm^3 ; for a particle size of 38 to 45 μm , 28 ± 3 μm and $(0.64 \pm 0.13) \times 10^7$ μm^3 ; for a particle size of 75 to 90 μm , 28 ± 4 μm and $(0.67 \pm 0.055) \times 10^7$ μm^3 ; and for a particle size of 125 to 150 μm , 35 ± 5 μm and $(0.81 \pm 0.19) \times 10^7$ μm^3 . Each BAG sample was statistically lower than that of the biofilms grown on the pure resin substrate, which had an average height of 53 ± 6 μm with a corresponding estimated volume of $(1.29 \pm 0.53) \times 10^7$ μm^3 . When the <5- μm BAG glass resin was compared with the pure resin sample, the 2 substrates were statistically different ($P < 0.05$). Comparing the 125- and 150- μm particle size resin with the pure resin substrate also showed a statistically significant difference. Within the range of the various particle sizes, those <5 μm and <38 μm were not statistically different at $P < 0.05$. Particle sizes <38 μm

Figure 2. Biofilm growth conditions. (A) Schematic of the biofilm growth setup. BAG resins were covered with Kapton tape, with a 2-mm diameter of the surface exposed, and then placed in the micro-incubator setup. Biofilms were grown for 72 h at 37°C in BMM solution with additions of 150 mM sucrose every 8 h. (B) Schematic of SECM setup. A 25- μm Pt microelectrode was used to map the biofilm morphology. Ag/AgCl and a 0.5-mm Pt wire were used as reference and counter electrodes, respectively. AS, artificial saliva; BAG, bioactive glass; BMM, basal medium mucin; SECM, scanning electrochemical microscopy.

Figure 3. Local chemical environment above BAG composites. (A) Average change in Ca^{2+} concentration (relative to artificial saliva) 20 μm above each BAG-containing resin composition in the presence of artificial saliva at pH 4.5. (B) Average change in pH at 20 μm above each BAG-resin composition in the presence of artificial saliva at pH 4.5. Values are presented as mean $\pm$ SD. BAG, bioactive glass.

and 38 to 45 μm were statistically different at $P < 0.05$, while particle sizes 38 to 45 μm and 75 to 90 μm were not. Particle sizes 75 to 90 μm and 125 to 150 μm were also statistically different at $P < 0.05$. Each biofilm sample was imaged in 3 different 500 $\times$ 500- μm areas (Fig. 4).

Discussion

In our previous study with pure BAG surfaces (not resin composites), a concentration of 700 μM of released Ca^{2+} was measured at 20 μm above the surface (Ummadi et al. 2016). The Ca^{2+} concentration value is about 3 times the maximum shown in the present study, which is in excellent agreement with the results from the current study for a composite having about one-third of its exposed surface being BAG particles. As all SECM experiments were performed in the presence of artificial saliva at pH 4.5, the local Ca^{2+} concentration results represented the amount of Ca^{2+} that can be released from these composites at pH 4.5. Thus, if these composites were exposed to a solution whose pH is >4.5, the local Ca^{2+} concentration would be significantly lower than the value reported here.

Figure 4. Representative image of biofilm morphology obtained with scanning electrochemical microscopy. BAG, bioactive glass.

Since the relationship between Ca^{2+} release and pH in dental materials has been well established (Xu et al. 2009; Xu and Moreau 2010; Weir et al. 2017), we did not conduct experiments at varying pH here. Such studies show that Ca^{2+} is released more rapidly and in greater amounts at low pH as compared with neutral pH. The purpose for using a solution at 4.5 was to simulate a worst-case condition under which the material would be exposed to an acid-producing biofilm in an active caries-forming environment, based on the fact that the pH can drop to as low as 4.9 (Takahashi and Nyvad 2016).

The average change in Ca^{2+} concentration and the corresponding pH change at a distance of 20 μm above the composites (Fig. 3A, B, respectively) showed that the composites containing BAG particle sizes $<5 \mu\text{m}$ and $<38 \mu\text{m}$ ($V_f = 0.32$) had the highest Ca^{2+} release, $224 \pm 99 \mu\text{M}$ and $213 \pm 148 \mu\text{M}$, respectively, suggesting that a BAG particle size $<38 \mu\text{m}$ ($V_f = 0.32$) may be the ideal composition in which to achieve maximum Ca^{2+} release and pH neutralization. Based on previous research in which a similar strep bacteria, *Streptococcus sobrinus*, was exposed to different concentrations of Ca ions, a very large killing effect was evident at 25 mM, with reductions of approximately one-half in colony counts at concentrations of 0.025 to 2.5 mM (Harris et al. 2016). This suggests that the concentrations achieved in the current study would be sufficient to have some effect on planktonic bacteria attempting to form a biofilm on these BAG-containing composites. There is the likelihood that the exposed BAG will dissolve and leave behind holes, and there is evidence for this in this literature (Beyth et al. 2008; Hyun et al. 2015). However, our previous study showed that the media actually had a larger effect than the biofilm in increasing surface roughness of a highly polished surface, as if the biofilm offered some protection, although surface hardness was reduced in both situations (Hyun et al. 2015).

None of the compositions, on average, showed more than a 0.2-unit pH change at a distance of 20 μm above the composites (Fig. 3B), as the amount of BAG present inside the resin composite was not sufficient to titrate the buffer to change the local pH. Our earlier studies (Ummadi et al. 2016) with pure BAG composites showed that the local pH shifts from 4.5 to 6.2 within 200 μm above the composite.

Thus, this study not only quantified the local metal ion concentration immediately above the composite but also correlated with what the bacteria would experience while growing on these composites, which is not achievable in traditional aliquot-based techniques.

To study the effect of the ion release on biofilm growth, we chose different BAG-resin composites with particle sizes of <5 to 150 μm . As shown in the Table, we used biofilm volume and height, as these better represent the amount of biofilm growing on different surfaces. The reduced height and volume of biofilm grown on the BAG-containing resin substrates as compared with the resin-only control indicate that the BAG particles incorporated into the resin matrix significantly reduced the growth of the bacterial biofilm as a result of the increased Ca^{2+} concentration. This was predictable based on the initial experiments measuring the release of calcium ions from the composites and by comparison of these concentrations with previous studies of similar bacteria exposed to lower and higher amounts of Ca^{2+} . The BAG composites failed to create a significant shift in pH in the local environment, yet biofilm formation was significantly suppressed on BAG-containing resins, indicating that changes in the Ca^{2+} concentration, rather than changes in pH, played the crucial role in inhibiting biofilm growth on these substrates. The finding that the calcium ion concentration was the more important factor in inhibiting biofilm growth is consistent with previous findings of BAG materials (Salehi et al. 2015).

Conclusion

Quantifying local ion concentrations is crucial in designing special ion-releasing dental composites capable of antimicrobial behavior, as the bacteria attempting to adhere to and subsequently grow on these composites may be affected by these ions within a few hundred micrometers above the surface. We observed that BAG particles with a diameter $<38 \mu\text{m}$ and a V_f of 0.32 in resin released the highest Ca^{2+} , with a local concentration of 224 μM in the presence of artificial saliva at pH 4.5. This pH would represent that present in a serious caries challenge created by an acid-producing biofilm on, or in the vicinity of, a tooth with a dental restoration. The same composite also showed significant inhibition of the growth of *S. mutans* biofilm on these surfaces over 3 d in the presence of BMM solution. Thus, this study helps correlate the local Ca^{2+} concentration released from the composites with the effects of Ca^{2+} on biofilm growth. This is possible through the use of our new SECM analytic technique, an outcome that would not otherwise be achievable through traditional assays. Further studies are ongoing in our laboratory to determine the local chemical environment at the interface of composites and biofilm.

Author Contributions

S. Aponso, contributed to data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; J.G. Ummadi, contributed to data acquisition, analysis, and interpretation, drafted the manuscript; H. Davis, contributed to data analysis, critically

revised the manuscript; J. Ferracane, contributed to conception, design, and data analysis, drafted and critically revised the manuscript; D. Koley, contributed to conception, design, and data acquisition, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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