Organo-Selenium-containing Dental Sealant Inhibits Bacterial Biofilm

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ABSTRACT

Oral bacteria, including S*treptococcus mutans* and *Streptococcus salivarius*, contribute to tooth decay and plaque formation; therefore, it is essential to develop strategies to prevent dental caries and plaque formation. We recently showed that organo-selenium compounds covalently attached to different biomaterials inhibited bacterial biofilms. Our current study investigates the efficacy of an organo-selenium dental sealant (SeLECT-Defense™ sealant) in inhibiting *S. mutans* and *S. salivarius* biofilm formation *in vitro*. The organo-selenium was synthesized and covalently attached to dental sealant material *via* standard polymer chemistry. By colony-forming unit (CFU) assay and confocal microscopy, SeLECT-Defense™ sealant was found to completely inhibit the development of *S. mutans* and *S. salivarius* biofilms. To assess the durability of the anti-biofilm effect, we soaked the SeLECT-Defense™ sealant in PBS for 2 mos at 37°C and found that the biofilm-inhibitory effect was not diminished after soaking. To determine if organo-selenium inhibits bacterial growth under the sealant, we placed SeLECT-Defense sealant over a lawn of *S. mutans*. In contrast to a control sealant, SeLECT-Defense™ sealant completely inhibited the growth of *S. mutans*. These results suggest that the inhibitory effect of SeLECT-Defense™ sealant against *S. mutans* and *S. salivarius* biofilms is very effective and durable.

KEY WORDS: organo-selenium, dental plaque, dental caries, dental sealant, SeLECT-Defense™, selenium-compounds.

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INTRODUCTION

Dental plaque is a biofilm that is produced by a bacterial community that may be composed of over 700 species (Kroes *et al*., 1999; Paster *et al*., 2001; Aas *et al*., 2005). One of the main components of this plaque is *Streptococcus mutans (S. mutans)*, which is also considered to be the primary etiologic agent of human dental caries and plaque formation (Hamada and Slade, 1980; Loesche, 1986). *S. mutans* is thought to play an important role in the development of tooth biofilm and caries by interacting with other *streptococci* (Kreth *et al*., 2005). *S. mutans* in particular produces glucosyltransferase enzymes that enable glucose to be transferred from sucrose for the synthesis of glucan (cellulose-like polymers), which can cause cariogenicity (Ogawa *et al*., 2011) and has been implicated in heart problems (Nakano *et al*., 2006).

Recently, it has been shown that an organo-selenium molecule served as a catalytic generator of superoxide radicals ($O_2^{\bullet-}$) (see below) from the oxidation of thiols (Seko and Imura, 1997). The superoxide radical appears to account for most of selenium's toxicity toward different bacteria, such as *Staphylococcus epidermidis, Staphylococcus aureus, Listeria monocytogenes, Salmonella typhimurium*, and *Escherichia coli in vitro* (Babior *et al*., 1975; Bortolussi *et al*., 1987; Kramer and Ames, 1988). We previously showed that organo-selenium covalently attached to different biomaterials and medical devices, such as intravenous catheter, contact lenses, and reverse osmosis (RO) membranes, as well as cellulose, blocks the formation of *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms (Mathews *et al*., 2006; Tran *et al*., 2009, 2012; Reid *et al*., 2010; Low *et al*., 2011). Low concentrations of organo-selenium (0.1% or 0.2%) were sufficient to inhibit bacterial attachment to these materials.

The catalytic mechanism by which selenium generates superoxide radicals has been previously described (Chaudiere *et al*., 1992). Organo-selenium ($R\text{-}Se^-$) donates an electron to oxygen-forming superoxide and then obtains an electron from ionized glutathione (GS^-), which is found in all body fluids. The net result from one catalytic cycle is 2 superoxide radicals, one glutathione disulfide and the original reduced organo-selenium.

In this study, we investigated the ability of organo-selenium polymerized into dental sealants to block *S. mutans* and *S. salivarius* biofilms *in vitro*.

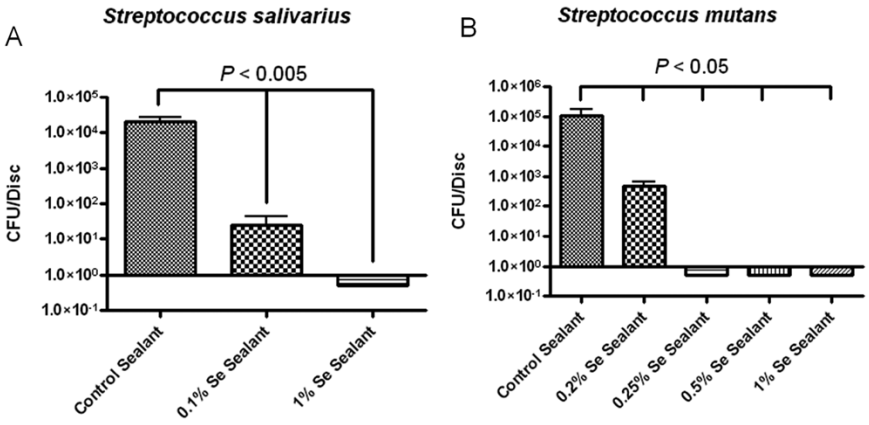

Figure 1. Effects of different doses of organo-selenium in dental sealants on biofilm development. Biofilms of either *S. salivarius* (**A**) or *S. mutans* (**B**) were allowed to form for 24 h on different sealants. Values represent the means of 6 or more independent experiments ± SD and represent CFU/7-mm sealant disc at different selenium concentrations. A one-way ANOVA with the Tukey-Kramer multiple-comparisons post-test, with the selenium-free dental sealant as the control, was done to determine statistical significance.

MATERIALS & METHODS

Bacterial Strains, Media, and Growth Conditions

S. salivarius strain ATCC® 13419 and *S. mutans* strain ATCC® 35668 were obtained from Remel (Lenexa, KS, USA). *S. salivarius* and *S. mutans* were routinely grown in Brain Heart Infusion (BHI).

Dental Sealant Materials

Selenium-containing dental sealants (SeLECT-Defense™ sealant; organo-selenium dental sealants) were obtained from Element-34 Technologies Inc. (Lubbock, TX, USA). The control selenium-free sealant was also obtained from Element-34. These were made into 7-mm discs. The sealant composition was: BisGMA, TEGDMA, and a multifunctional monomer for methacrylate formation, with 0.096% camphorquinone. The selenium compound, 3-[3-((2-{1-methyl-2-[2-(2-methyl-acryloyloxy)-ethoxycarbonyl]-ethoxycarbonyl}-ethyldiselenyl)) -propionyloxy]-butyric acid 2-(2-methyl-acryloyloxy)-ethyl ester, is a diselenyl-methacrylate.

In vitro Biofilm Systems

Quantitative Analysis of the Biofilms (CFU/segment)

The biofilm assay was performed, as previously described, by a modified microtiter plate assay (Hammond *et al*., 2010; Tran *et al*., 2012). Seven-millimeter discs of selenium-free dental sealant or organo-selenium dental sealants were incubated in 1 mL BHI media in the presence of approximately 1,000 initial colony-forming units (CFU) of *S. salivarius* or 7,500 CFU of *S. mutans* in each well of the microtiter plate. The plates were incubated under micro-aerobic conditions, which were generated when the plate was placed inside a gas jar containing an EZ GasPak (Catalog no. 260678, BD, Franklin Lakes, NJ, USA) at 37°C for 24 hrs. Biofilms were quantified by determination of the CFU *per* dental sealant disc. Each piece was carefully removed from the well, rinsed gently with sterile distilled H_2O, and placed in a microcentrifuge tube containing 1 mL of phosphate-buffered saline (PBS). The tubes were placed in a water bath sonicator for 10 min to loosen the cells within the biofilm and then vigorously vortexed 3 times for 1 min to detach the cells. Suspended cells were serially diluted 10-fold in PBS, and 10-µL aliquots of each dilution were spotted onto Tryptic Soy Agar plates, with 5% sheep blood. The plates were incubated at 37°C for 24 h, and the CFU were counted according to the following formula: CFU x dilution factor x 100. To confirm the efficacy of our protocol for recovery of biofilm-associated bacteria from each dental sealant disc, we washed the discs 3 times with sterile PBS, placed them in fresh tubes containing PBS, and repeated the sonication and vortexing process. We did not recover CFU from plating of the PBS wash after the new sonication (data not shown), nor did we visualize residual bacteria by scanning electron microscopy on the vortexed segments.

Biofilm Analysis by Confocal Laser Scanning Microscopy (CLSM)

This was prepared as described above in Materials & Methods by the microtiter plate assay.

Biofilms formed by *Streptococcus mutans* were stained by means of the BacLight™ Bacterial Viability kit (Molecular Probes, Eugene, OR, USA) as previously described (Hammond *et al*., 2011). Three control and three Se-sealant disks were examined for the presence of biofilm by confocal laser scanning microscopy (CLSM). Visualization of the biofilms was accomplished with an Olympus IX71 Fluo-view 300 confocal laser scanning microscope (Olympus America, Melville, NY, USA). Three-dimensional biofilm image reconstructions were performed with NIS-Elements 2.2 software (Nikon) as previously described (Nidadavolu *et al*., 2012).

The biofilm structural features were analyzed with the COMSTAT program (Heydorn *et al.*, 2000). We obtained several image stacks of each biofilm by CLSM, and the images were analyzed as previously described (Tran *et al*., 2012).

Analysis of Bacterial Growth under the Sealant

The ability of *Streptococcus mutans* to grow under Se-sealants was determined by a modification of the agar diffusion method as described previously (Baue, *et al*., 1966). Overnight cultures of *Streptococcus mutans* were diluted with BHI broth to an absorbance at 600 nm, 0.5 of which matches the 0.5 McFarland standard (National Committee for Clinical Laboratory Standards, 1990, 1993). A lawn of *Streptococcus mutans* was prepared on a BHI agar plate, as previously described (National Committee for Clinical Laboratory Standards, 1990, 1993), and 7-mm sealant

disks were placed individually, with sterile forceps, onto the bacterial lawns. The plates were incubated under micro-aerobic conditions at 37°C for 24 hrs. The resulting zones of inhibition were measured in millimeters, and the areas under the sealant disks were also examined for growth inhibition. Images were taken before and after the sealant disks were removed. In addition, sealant disks were also examined by the CFU assay for bacteria attached to the sealant.

Analysis of the Long-term Stability of the Organo-Selenium-containing Sealant

Seven-millimeter discs of the OS dental sealants were completely immersed in 10 mL PBS in sterile glass tubes and incubated at 37°C for 2 mos. At the end of the incubation period, the pieces were removed, dried, and utilized in the *in vitro* biofilm assay, as described above, for determination of the durability of the coating. They were compared with Se-sealant, which was incubated under ambient conditions.

Statistical Analyses

Results of the CFU assays were analyzed with Prism® version 4.03 (GraphPad Software, San Diego, CA, USA) with 95% confidence intervals (CIs) of the difference. Comparisons of the *in vitro* biofilms formed on selenium-free and organo-selenium dental sealants were analyzed by one-way analysis of variance (ANOVA) with the Tukey-Kramer multiple-comparisons post-test and a two-tailed unpaired *t* test, to determine significant differences. Each experiment had 6 samples. In addition, each experiment was repeated several times, and similar results were obtained. The only exception to this was the results shown in the Biofilm Visualization section later, which represent experiments done twice in triplicate.

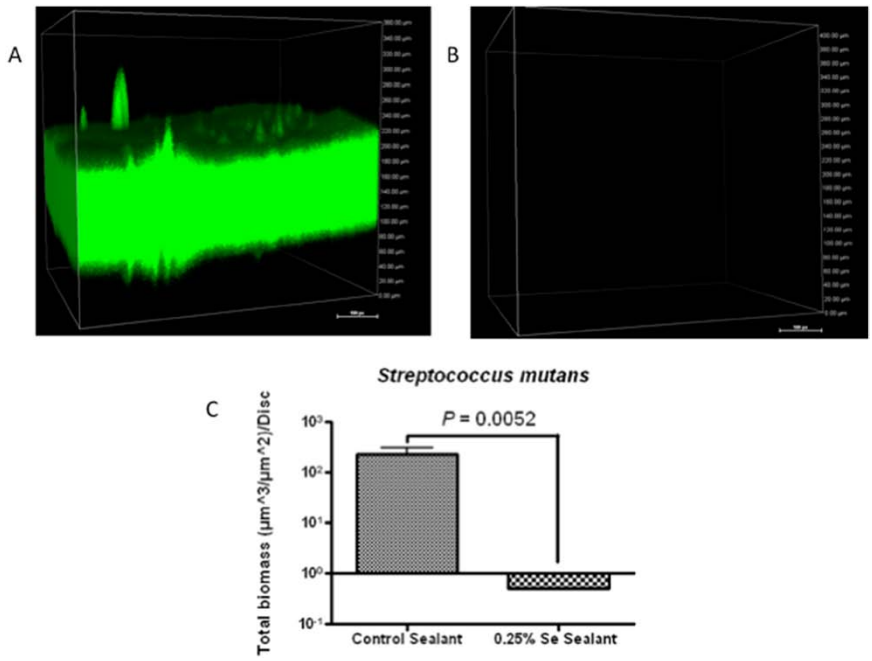

Figure 2. Visualization of *S. mutans* biofilm formation on control dental sealant and organo-selenium dental sealant (0.25% selenium) *in vitro* by confocal laser scanning microscopy. Biofilms formed by *S. mutans* were stained with the BacLight™ Bacterial Viability kit, which contains fluorescent dye, Syto-9™, which stains live bacteria green. Three-dimensional analysis of the images (A, B): Bars, 100 pixels; box dimensions, width 800 pixels, height 600 pixels, depth 360 µm (**A**) and 400 µm (**B**). (A) Image of biofilm on the control sealant. (B) Image of biofilm on the organo-selenium sealant. (**C**) COMSTAT analysis of confocal data for the total biomass $\mu m^3/\mu m^2$ on the control and selenium sealants. A two-tailed unpaired *t* test with the selenium-free discs as the control was conducted to determine statistical significance.

RESULTS

Organo-Selenium Dental Sealants Inhibit the Development of *S. salivarius* and *S. mutans* Biofilms on Sealant *in vitro*

We examined the ability of *S. salivarius* and *S. mutans* to form biofilms on selenium-free dental sealant discs, using the modified microtiter biofilm assay (Hammond *et al*., 2010; Tran *et al*., 2012). We recovered an average of 4 x 104 CFU/sealant disc for *S. salivarius* and an average of 2 x 105 CFU/sealant disc for *S. mutans*, using as control a selenium-free sealant (Figs. 1A, 1B).

We then tested the efficacy of dental sealant discs containing different concentrations of selenium to inhibit *S. salivarius* and

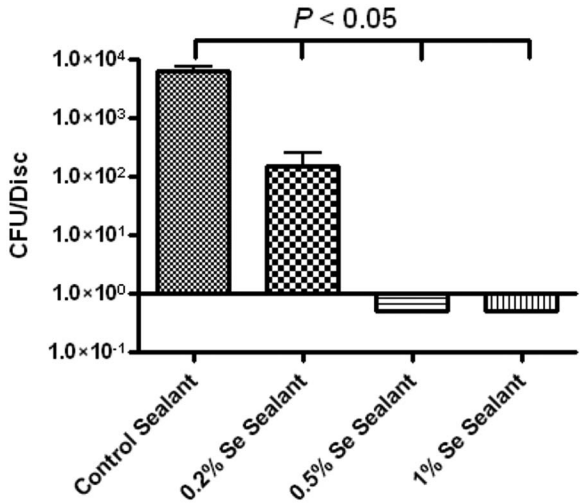

Figure 3. Determination of the stability of organo-selenium dental sealants. Organo-selenium dental sealants and control sealants were soaked in PBS for 2 mos and tested with *S. mutans*. Biofilms were allowed to form for 24 hrs. Values represent the means of 3 or more independent experiments ± SD (6 samples *per* experiment). A one-way ANOVA with the Tukey-Kramer multiple-comparisons post-test with the selenium-free discs as the control was done to determine statistical significance.

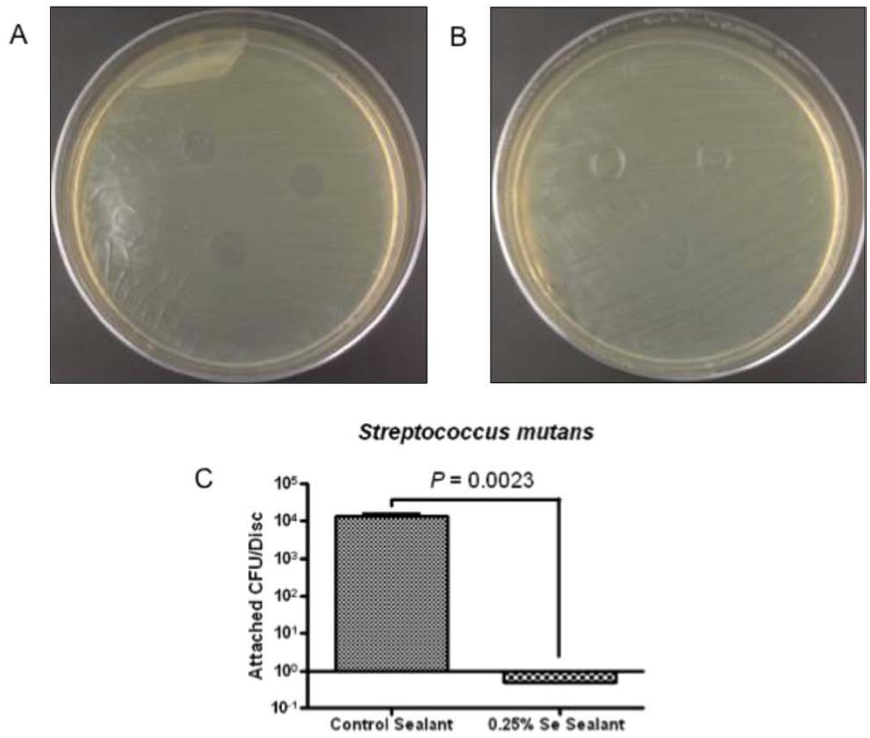

Figure 4. Determination of the ability of selenium sealants to inhibit bacterial growth under the sealant. Sealants were placed on a bacterial lawn of *S. mutans*. (**A**) 0.25% selenium sealant. (**B**) Control sealant. (**C**) CFU determination of bacteria growing on the control and selenium sealant after 24 hrs on the bacterial lawn. A two-tailed unpaired *t* test with the selenium-free discs as the control was done to determine statistical significance.

S. mutans biofilms. Dental sealant discs containing 1% organo-selenium inhibited the development of *S. salivarius* biofilms (no CFU recovered). In addition, and in comparison with the control selenium-free dental sealant discs, we obtained significantly fewer ($p < .05$) CFUs of *S. salivarius* for sealants containing 0.1% selenium (Fig. 1A). For *S. mutans*, dental sealant discs containing either 1%, 0.5%, or 0.25% selenium completely inhibited the development of *S. mutans* biofilms (Fig. 1B). In comparison with selenium-free dental sealant discs, dental sealants containing 0.25% organo-selenium reduced the development of *S. mutans* biofilms significantly ($p < .05$). The results indicate that even relatively low concentrations of selenium (0.25%) are efficient in inhibiting biofilm formation by both *S. salivarius* and *S. mutans*.

Biofilm Visualization

To confirm the above results, we visualized *S. mutans* biofilms using confocal laser scanning microscopy (CLSM). The biofilms were developed as described above. On selenium-free dental sealant discs, *S. mutans* formed typical, mature, and well-developed biofilms (Fig. 2A). No bacteria were detected on the 0.25% Se-sealant discs (Fig. 2B). Using the COMSTAT program, we analyzed the biofilms' structure. The control sealant showed biofilms with a biomass of 315 $\mu m^3/\mu m^2$, an average thickness of 429 μm, and a surface area of 47 x 10^6 μm^2, while the organo-selenium sealant showed values of zero for all 3 categories (Figs. 2C). As can be seen, a mature biofilm forms on the selenium-free sealant and not on the organo-selenium sealant. We obtained similar results using sucrose-containing BJI as a biofilm medium (data not shown).

Organo-Selenium Dental Sealants' Stability

Sealant discs were immersed in PBS for 2 mos at 37°C. Compared with selenium-free dental sealants, total inhibition was obtained with both 1% and 0.5% sealants (Fig. 3). A similar result was obtained with sealants that were incubated under ambient conditions.

Inhibitory Effects of Organo-Selenium Dental Sealants to Bacteria Growing under the Sealant

We found that organo-selenium sealant placed upon a lawn of *S. mutans* completely inhibited the growth of bacteria under the organo-sealant (Fig. 4A), while a non-selenium sealant had no effect (Fig. 4B).

DISCUSSION

A 2008 review of electronic databases of comparative studies examining bacteria levels in sealed permanent teeth showed that sealants reduced bacteria in caries lesions, but some studies reported that low levels of bacteria persisted (Oong *et al*., 2008). With this in mind, we carried out the present study to investigate the effectiveness of bacterial attachment and biofilm formation on the surfaces of organo-selenium dental sealants *in vitro*.

Several previous studies indicated an antimicrobial effect for fluoride-containing dental sealants against oral bacteria (Loyola-Rodriguez and Garcia-Godoy, 1996; Matalon *et al*., 2003; Naorungroj *et al*., 2010). However, these studies primarily examined the antibacterial effect of the fluoride released from these dental sealants. In our present study, we examined the ability of organo-selenium dental sealants to inhibit bacterial attachment and biofilm development by *S. mutans* and *S. salivarius* directly on the dental sealants. In this regard, our assay closely resembles an *in vivo* situation where bacterial biofilm (plaque) develops directly on the dental sealants. As shown in Figs. 1 and 2, organo-selenium dental sealants inhibited *S. mutans* and *S. salivarius* biofilm development on the surfaces of organo-selenium dental sealants. In addition, we examined the ability of

bacteria to grow under the sealants. We detected no bacterial growth under either the organo-selenium sealant (Fig. 4) or on the sealant itself.

The other advantage of the organo-selenium dental sealants over fluoride-containing sealants is the stability of the antibacterial effect. As shown by previous studies, the gradual release of fluoride compromises the antibacterial effect of the fluoride-containing sealants (Loyola-Rodriguez and Garcia-Godoy, 1996; Matalon *et al*., 2003; Naorungroj *et al*., 2010). As a result, over time, the efficiency of these dental sealants would be compromised, which necessitates the recharge of more fluoride salt to these sealants to enhance their antimicrobial activity. In contrast, after 2 mos of soaking in PBS, organo-selenium dental sealants still retained their antibacterial effect and significantly inhibited *S. mutans* attachment and biofilm formation (Fig. 3). Because of the covalent attachment of selenium to the polymer of the sealant, only very small amounts of unpolymerized selenium may be released from the sealant.

The significant difference between bacterial attachment and biofilm formation on control dental sealants *vs*. organo-selenium dental sealants is likely to be due to the $O_2^{\bullet-}$, catalytically produced by the organo-selenium, which causes oxidative stress that damages the bacterial cell walls and DNA (Babior *et al*., 1975; Fridovich, 1985; Bortolussi *et al*., 1987; Kramer and Ames, 1988). Also, we have shown, in a previous study, that organo-selenium in polymer form can generate superoxide radicals (Tran *et al*., 2009).

As for bacteria living under the selenium sealant, our studies also showed that bacteria can survive under regular sealant but not under the Se-sealant (Fig. 4). Thus, the selenium sealant would not allow bacteria to survive and to form dental caries under the sealant. This is in spite of the fact that we have previously found that the selenium-killing mechanism is much slower in a low-oxygen environment.

In conclusion, we have studied a new method for covalently incorporating an antibacterial agent into dental sealants. Organo-selenium polymerized into dental sealant is effective in inhibiting bacterial attachment and biofilm formation by the two main oral pathogens, *S. mutans* and *S. salivarius*. In addition, the organo-selenium dental sealant is very durable in that it is still completely effective in inhibiting *S. mutans* attachment after 2 mos of soaking in aqueous solution. Therefore, the use of organo-selenium dental sealants has the potential to prevent dental caries and plaque formation by oral pathogens. In addition, bacteria cannot live under the dental sealant, while they can live under a control sealant. Results of our recent clinical trials confirmed that organo-selenium sealant inhibits bacterial plaque formation on human teeth (Amaechi *et al*., unpublished observations).

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