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<th><strong>Title</strong></th>
<th>Protection offered by root-surface restorative materials against biofilm challenge</th>
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INTRODUCTION

Dental caries and periodontal disease are associated with changes in the metabolism and composition of the oral flora at specific sites. Because conditions within the mouth are never stable for long periods, many in vivo environmental conditions are difficult to control and manipulate. Although biofilms in in situ studies have been reported to be consistent within an individual, they varied significantly among individuals (Arweiler et al., 2004; Moura et al., 2004). In vitro studies can be advantageous, because most of the environmental conditions and the microbiota can be controlled and changed (Sissons, 1997).

The characteristics of biofilms formed by major cariogenic microorganisms in the artificial mouth have been shown to be similar to those of dental plaque on the surfaces of roots with caries (Shu, 1988). When a biofilm is allowed to form on enamel and dentin in vitro, the demineralization observed is similar to that in a natural caries lesion (Shu et al., 2000). Fontana et al. (2004) showed that biofilm development was associated with 5 cariogenic micro-organisms and the depth of demineralization in enamel. They also found that, although sucrose 'feeding time' did not affect lesion size, the frequency of sucrose feeding did.

There has so far been no study of the effects of mixed-species oral biofilms formed by major cariogenic microorganisms in the artificial mouth. The prevalence of root-surface caries is increasing. We hypothesized that some restorative materials are protective against cariogenic challenge on root surfaces. Our goal was to study the effects of different restorative materials on root surfaces incubated with an oral biofilm generated in an artificial mouth. A biofilm of Streptococcus mutans, Streptococcus sobrinus, Lactobacillus rhamnosus, and Actinomyces naeslundii was co-cultured for 21 days on 24 glass-ionomer cement, resin-modified glass-ionomer cement, or resin-composite-restored root surfaces. These surfaces were then examined with Fourier transform infrared spectroscopy and scanning electron energy-dispersive spectroscopy. Only glass-ionomer restorations showed a significant increase in log calcium-to-phosphorus ratio (P < 0.01), and a significantly lower log amide I-to-hydrogen phosphate ratio on the root surface after incubation in the artificial mouth. Glass-ionomer restoratives conferred a preventive effect on the root surfaces against initial cariogenic challenge with a mixed-species oral biofilm without therapeutic intervention.

KEY WORDS: artificial mouth, oral biofilm, restorative materials, root surface, caries.

MATERIALS & METHODS

Restorative Materials

Three restorative materials were compared: resin composite, resin-modified glass-ionomer cement, and glass-ionomer cement. The types, compositions, and fluoride-release and fluoride-depletion times of restoratives used are given in the Table.

Biofilm Formation on Restored Root Surfaces

Twenty-four sound, extracted human third molars were supplied by the oral and maxillofacial surgery unit of the Prince Philip Dental Hospital, The University of Hong Kong. The use of human tissues followed an approved protocol that satisfied...
the requirement of the IRB, Faculty of Dentistry, The University of Hong Kong, and informed patient consent was obtained.

One cavity (3 x 3 x 3 mm³) was prepared midway across the enamel-cementum junction in each of the 24 teeth selected, with 6 teeth in each of the 3 restorative groups. The restored teeth were individually bottled and aged in water (replenished) at room temperature for 3 mos, to allow the fluoride content to be depleted and absorbed into the enamel and root sides adjacent to the restorations. The aged teeth were then sectioned into cubes containing the restoration (each side measuring 5 mm), by means of a diamond-impregnated disc (Isomet; Buehler Ltd., Lake Bluff, IL, USA) under water cooling. Two of each type of restorative material were randomly assigned to 1 microstation, and 6 tooth specimens in each of the 3 restorative groups. The restored teeth were sectioned midway across the restoration, along the long axis of the restored tooth specimen. One half of the specimen was used for scanning electron energy-dispersive spectroscopy (SEM-EDS). The root/enamel sides were compared because the lesion was created on both sides of the restorative materials.

### Scanning Electron Microscopy

The objective of SEM-EDS was to study the changes in mineral content (in terms of log calcium-to-phosphorus Ca:P ratio) of calcified tooth tissue in the demineralized area under the biofilm generated on the restored root surface in the artificial mouth. The restored tooth specimens were first prepared and examined under a scanning electron microscope (Gemini, Leo 1530, Germany) set at 20 kV. An assessment of the log Ca:P of demineralized and sound areas adjacent to the demineralized areas was made by energy-dispersive spectroscopy (model 7426; Oxford Instruments, Oxford, UK). Elemental analysis was carried out across the root surface at the restorative margin of the enamel in 3 line scans that were 30 μm apart, with the 1st line being 30 μm from the tooth-restoration margin. Four blocks were placed in 4 biofilm holders, each housed in microstations of an artificial mouth culture system. Under computer control, sucrose (5%, w/v) was supplied continuously at 0.2 mL/min throughout the experimental period (Wong and Sissons, 2001). Biofilms were generated on the restored root surface in the artificial mouth. The restored tooth specimens were first prepared and examined under a scanning electron microscope (Gemini, Leo 1530, Germany) set at 20 kV. An assessment of the log Ca:P of demineralized and sound areas adjacent to the demineralized areas was made by energy-dispersive spectroscopy (model 7426; Oxford Instruments, Oxford, UK). Elemental analysis was carried out across the root surface at the restorative margin of the enamel in 3 line scans that were 30 μm apart, with the 1st line being 30 μm from the tooth-restoration junction. Each line scan consisted of 10 points, 20 μm apart (total of 10 x 3 x 5 = 150 spot analyses for each restorative material tested and 150 internal controls for each group, giving 300 analyses).

### Table. Characteristics of Restorative Materials Used

<table>
<thead>
<tr>
<th>Tooth Tissue or Restorative</th>
<th>Manufacturer</th>
<th>Shade</th>
<th>Composition</th>
<th>Wavenumber (cm⁻¹)</th>
<th>Chemical Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resin composite</td>
<td>3M-ESPE,</td>
<td>A3</td>
<td>Bisphenol-A-polymethylene glycol-diether dimethacrylate, trimethylene glycol dimethacrylate, zirconium oxide, silica fillers (4%), w/w, photoinitiator (trace).</td>
<td>837</td>
<td>C-H &quot;oop&quot; in aromatics (900-675)</td>
</tr>
<tr>
<td>Filltek Supreme (syringe)</td>
<td>St. Paul, MN, USA</td>
<td></td>
<td>Fluoride release rates (Vermeersch et al., 2001): 1-day: 0.00 μg/mm²/day, 90-day: 0.00 μg/mm²/day</td>
<td>1265</td>
<td>C-O-C stretch (1250-1310)</td>
</tr>
<tr>
<td>Resin-modified glass-ionomer cement Photac-Fil (capsule)</td>
<td>3M-ESPE, Seefeld, Germany</td>
<td>A3</td>
<td>Powder: Sodium-calcium-aluminum-fluoro-silicate glass Liquid: (1) Acrylic and maleic acid copolymer (2) Glass-ionomer compatible monomer and oligomer (3) Camphor quinone (4) Water Fluoride release rate (Vermeersch et al., 2001): 0-day: 0.13 μg/mm²/day, 90-day: 0.00 μg/mm²/day</td>
<td>796</td>
<td>C=H bend in alkenes (1000-650)</td>
</tr>
<tr>
<td>Glass-ionomer cement</td>
<td>3M-ESPE,</td>
<td>A3</td>
<td>Powder: calcium aluminum-lanthanum-fluoro-silicate glass, acrylic acid-maleic acid copolymer, pigments Liquid: water, acrylic acid-maleic acid copolymer, tartaric acid Fluoride release rate (Vermeersch et al., 2001): 0-day: 1.05 μg/mm²/day, 90-day: 0.00 μg/mm²/day</td>
<td>1217</td>
<td>C-O stretch in esters (1310-1250)</td>
</tr>
<tr>
<td>Ketac-Molar Applicap (capsule)</td>
<td>Seefeld, Germany</td>
<td></td>
<td></td>
<td>1450</td>
<td>C-H stretch in alkanes (1470-1450)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1685</td>
<td>C=O stretch in alpha, beta-unsaturated aldehydes (1710-1665)</td>
</tr>
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37°C and 100% humidity. At the end of day 21, the bacterial compositions of the inoculum and the biofilm were analyzed. Gram stain, catalase test results, and total microbiological counts confirmed the bacterial species similarity of the oral biofilms (Shu et al., 2000; Wong and Sissons, 2001). Each tooth sample was sectioned midway across the restoration, along the long axis of the restored tooth specimen. One half of the specimen was used for Fourier transform infrared (FTIR) spectroscopy, and the other was used for scanning electron energy-dispersive spectroscopy (SEM-EDS). The root/enamel sides were compared because the lesion was created on both sides of the restorative materials.
Fourier Transform Infrared Spectroscopy

Any changes in the chemical structure of the restored tooth surface were analyzed by UMA-500 detector-equipped microscopy (Bio-Rad Laboratories, Hercules, CA, USA). The infrared radiation used ranged from 650 to 4000 cm\(^{-1}\) in wavelength number. The FTIR spectrum of restored tooth specimens (n = 5 for each restorative tested) was obtained by the average acquisition of data at the spatial resolution achieved with a 100 x 100 \(\mu\)m aperture.

This was based on a technique used to measure the difference between demineralized and remineralized bone (Mythili et al., 2000). The organic matrix of dentin and bone is composed mainly of type I collagen (resulting in an amide band in the FTIR spectrum), and the mineral matrix is composed of nearly the same amount of biological apatite in dentin (\(\text{HPO}_4^{2-}\) band in the FTIR spectrum) (Magne et al., 2001). The changes in mineral content were calculated on the basis of the spectrally derived matrix-to-mineral ratio (the areas of absorbance of the protein amide I peak between 1585 and 1720 cm\(^{-1}\) to the phosphate (\(\text{HPO}_4^{2-}\)) peak between 900 and 1200 cm\(^{-1}\)).

Statistical Analysis

The differences between the mineral densities were assessed by one-way analysis of variance (ANOVA). We used a post hoc Tukey test to detect any significant differences between demineralized areas and sound areas in the same specimens (internal controls). Analyses were performed with SPSS software (version 13.0, SPSS Inc., Chicago, IL, USA). A 5% significance cut-off level was used for all analyses.

RESULTS

Analysis of Biofilm Bacteria

The microbiological tests showed that the micro-organisms at the end of the 21-day experimental period were similar, and the bacterial loading of the oral biofilm was in the moderate range of 0.35-3.4 x 10\(^8\). The predominant streptococcal species was \textit{S. mutans} after growth competition with \textit{S. sobrinus}. The aged restoration showed negligible fluoride release (90-day: 0.00 \(\mu\)g/mm\(^2\)/day; Vermeersch et al., 2001) (Table) and did not seem to have any effect on the levels of \textit{S. mutans} or other bacteria in the oral biofilm.
the log [amide I: HPO$_4^{2-}$] on the root surfaces of glass-ionomer restorations was significantly lower than those of the other 2 materials (P = 0.04) (Fig. 3), and that the log [amide I: HPO$_4^{2-}$] of the demineralized surface was lower than that of the control sound tooth surface (P = 0.03).

DISCUSSION

Our study simulated a high-caries-risk situation where oral biofilm received no intervention from oral therapeutic agents for a 21-day experimental period. The findings showed that fluoride-depleted glass-ionomer cement conferred a therapeutic and preventive effect on the root surface, but not the enamel, against initial cariogenic challenge from a mixed-species oral biofilm generated in an artificial mouth. The anticariogenic effect of the glass-ionomer cement may be related to its ability to inhibit demineralization by fluoride release. However, fluoride-depleted resin-modified glass-ionomer cement also released fluoride, but did not confer a therapeutic or preventive effect on either side of the restored root surface. However, we cannot eliminate the possibility of an antimicrobial effect on the restorations from substances other than fluoride. We previously demonstrated that one glass-ionomer cement showed the penetration of strontium and fluoride into dentin (Smale et al., 2005).

Fluoride-releasing restorative materials have been found to inhibit demineralization of the enamel and root sides of the root surface (Lam et al., 1998; Gonzalez Ede et al., 2004). Interestingly, Sa et al. (2004) showed that glass-ionomer cement was not anticariogenic in human root dentin cultured in a microbial model with S. mutans and L. casei, but did show anticariogenic properties in a chemical-demineralizing model. Different combinations of cariogenic oral micro-organisms indeed affect the demineralization of the root surface differently (Shen et al., 2004).

We observed a drop in transmittance and peak area intensity across the FTIR spectrum of glass-ionomer cement and, to a lesser extent, across that of resin-modified glass-ionomer cement after the restored root surfaces had been cultured for 3 wks under a biofilm generated in an artificial mouth. In contrast, resin composite was not affected. These results differed from those observed when an artificial saliva system was used (Yip and To, 2005), perhaps due to the different conditions of artificial saliva and mixed-species oral biofilm.

Enamel and dentin are composed of an organic matrix, a mineral matrix, and water (Bachmann et al., 2003). In enamel tissue, 2 infrared signals from the hydroxyl group can be observed: at 3570 cm$^{-1}$, associated with stretching, and at 749 cm$^{-1}$, associated with vibration. Root-surface and dentin specimens have profiles showing basically the same bands that differ only in their proportions—that is, the amide peaks are higher in root-surface profiles than in dentin profiles (Sasaki et al., 2002).

Presumably, acids from the oral biofilm dissolve hydroxyapatite (HAP) and expose the previously HAP-masked collagens and organic matrices, thereby generating more carbonyl groups (Di Renzo et al., 2001a). In addition, exposed collagen will be quickly attacked by bacterial proteolytic enzymes (Di Renzo et al., 2001b). The altered matrix of the root side of the restored root surface of a glass-ionomer restoration was shown to be least susceptible to demineralization by the cariogenic challenge of a mixed-species oral biofilm.
biofilm generated in an artificial mouth, possibly due to the conversion of the hydroxyapatite to fluoroapatite on the root surface during the process of fluoride absorption from the restorative material. The preventive and therapeutic effects of glass-ionomer restorations may be explained by increased resistance to removal of fluoroapatite by acid on the root side, because of its significantly higher mineral content (higher log Ca:P) compared with the other restorative materials tested. Therefore, less collagen and organic matrix was exposed (lower log [amide I to HPO4^2-]) to cariogenic challenge by the mixed-species oral biofilm. All restorative materials tested did not significantly alter the mineral content and organic matrices on the enamel side of the restored root surface.

Our findings show that it is necessary to differentiate between cavities on the enamel and root sides of a restored root surface, because the preventive effect of glass-ionomer cement is restricted to the root side.

Glass-ionomer cement was the only restorative material of the 3 tested that conferred a preventive and therapeutic effect on the root surface against initial cariogenic challenge by mixed-species oral biofilm generated in an artificial mouth, simulating a high-caries-risk situation with no oral therapeutic intervention. The first null hypothesis—that restorative materials have no effect on the root surface under the oral intervention. The first null hypothesis—that restorative materials confer a preventive and therapeutic effect on the enamel and root surface—is also rejected.

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