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Protection Offered by Root-surface Restorative Materials against Biofilm Challenge

ABSTRACT

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.

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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 micro-organisms 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 micro-organisms on the surfaces of restored roots, despite the increased prevalence of root-surface caries (Griffin *et al.*, 2004). The objective of this study was to conduct an elemental analysis of the mineral content of the surfaces of restored roots under a simulated oral biofilm generated in an artificial mouth culture system. Fluoride has been shown to move across the biofilm *in vivo* (Watson *et al.*, 2005), so this study used fluoride-depleted specimens to minimize the effect of fluoride diffusion in the biofilm between the surface of the restorative material and the root surface. The first null hypothesis was that restorative materials have no effect on the root surface under the oral biofilm generated in an artificial mouth. The second null hypothesis was that restorative materials confer the same therapeutic effect on the enamel and root surface.

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

Table. Characteristics of Restorative Materials Used

Tooth Tissue or Restorative	Manufacturer	Shade	Composition	Wavenumber (cm^{-1})	Chemical Group (range, cm^{-1})
Resin composite Filtek Supreme (syringe)	3M-ESPE, St. Paul, MN, USA	A3	Bisphenol-A-polyethylene glycoldiether dimethacrylate, trimethylene glycol dimethacrylate, zirconium oxide, silica fillers (4%, w/w), photoinitiator (trace).	837	C-H "oop" in aromatics (900-675)
				1265	C-O-C stretch 1250-1310
			Fluoride release rates (Vermeersch *et al.*, 2001): 1-day: 0.00 μg/mm²/day	1741	C=O stretch in esters (1750-1735)
			90-day: 0.00 μg/mm²/day	1870	Unknown
Resin-modified glass-ionomer cement Photac-Fil (capsule)	3M-ESPE, Seefeld, Germany	A3	Powder: Sodium-calcium-aluminum-fluoro-silicate-glass Liquid: (1) Acrylic and maleic acid copolymer	796	=C-H bend in alkenes (1000-650)
			(2) Glass-ionomer compatible monomer and oligomer	1220	C-O stretch in esters (1310-1250)
			(3) Camphor quinone (4) Water	1735	C=O stretch in esters (1750-1735)
			Fluoride release rate (Vermeersch *et al.*, 2001): 0-day: 0.13 μg/mm²/day 90-day: 0.00 μg/mm²/day		
Glass-ionomer cement Ketac-Molar Applicap (capsule)	3M-ESPE, Seefeld, Germany	A3	Powder: calcium aluminum-lanthanum-fluorosilicate glass, acrylic acid-maleic acid copolymer, pigments	1217	C-O stretch in esters (1310-1205)
			Liquid: water, acrylic acid-maleic acid copolymer, tartaric acid	1450	C-H stretch in alkanes (1470-1450)
			Fluoride release rate (Vermeersch *et al.*, 2001): 0-day: 1.05 μg/mm²/day 90-day: 0.00 μg/mm²/day	1685	C=O stretch in alpha, beta-unsaturated aldehydes (1710-1665)

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 mm^3) 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 total were embedded in 1 epoxy resin block at 60°C for 48 hrs (TAAB 812 resin; TAAB Laboratories, Aldermaston, UK). The surfaces of each epoxy resin block were polished with 600-grit sandpaper to give a flat surface, and the blocks were sterilized overnight with 2% glutaraldehyde. 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 every 8 hrs for 6 min, and the basal medium mucin (BMM) was supplied continuously at 0.2 mL/min throughout the experimental period (Wong and Sissons, 2001). Biofilms were created on tooth specimens with 4 bacterial species—namely, *Streptococcus mutans, Streptococcus sobrinus, Lactobacillus rhamnosus,* and *Actinomyces naeslundii* (Shu *et al.*, 2000). Bacterial inoculation was performed on days 1, 3, and 5 to facilitate the establishment of all bacterial species, which were incubated at 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.

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 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).

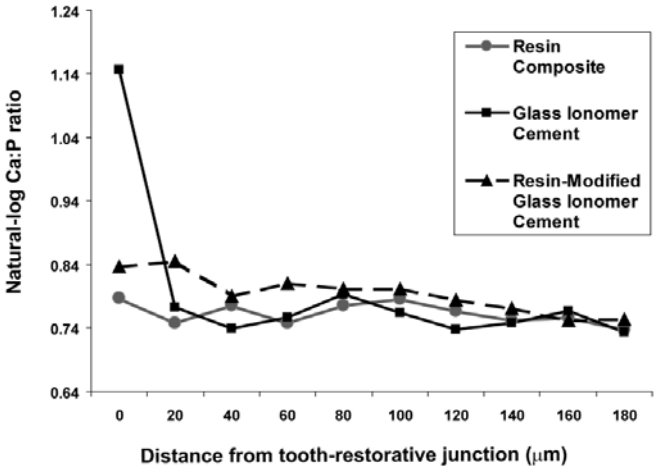

Figure 1. Mineral density (log Ca:P) of the restored root surface after 3 weeks' culture with oral biofilm. The log Ca:P was measured by energy-dispersive spectroscopy from the root surface to 200 μm in depth (total of 10 x 3 x 5 = 150 spot analyses for each restorative material tested and 150 internal controls for each group, giving 300 analyses). Glass-ionomer cement was the only material to show an increase in log Ca:P at the root surface adjacent to the restoration ($P < 0.01$). The log Ca:P tailed off at distances farther from the interface. Such results were not found at the enamel surface.

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 μm 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 (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 (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 *S. mutans* after growth competition with S. sobrinus. The aged restoration showed negligible fluoride release (90-day: 0•00 μg/mm^2/day; Vermeersch *et al.*, 2001) (Table) and did not seem to have any effect on the levels of *S. mutans* or other bacteria in the oral biofilm.

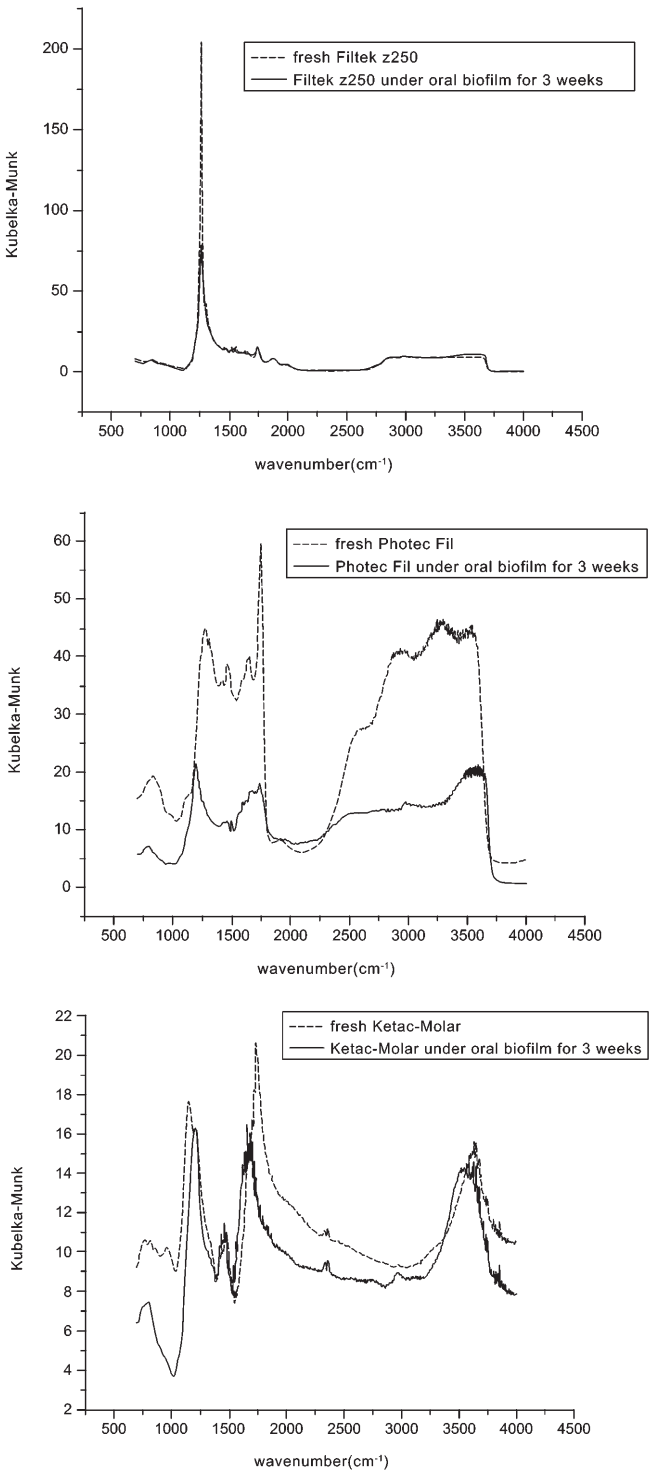

Figure 2. FTIR spectra of the restorative materials used.

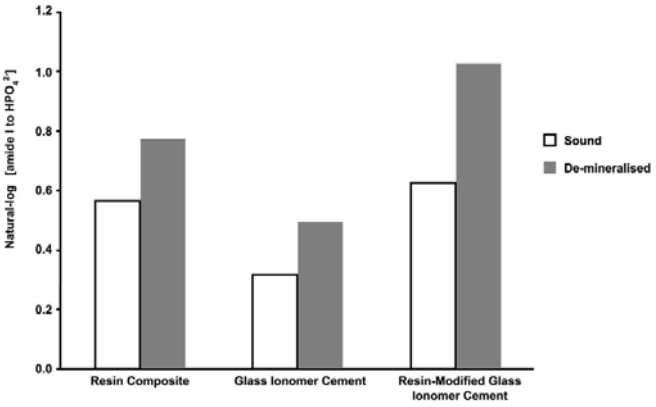

Figure 3. Log FTIR intensity ratios of amide I to HPO_4^{2-} showing the mineral content of restored enamel and restored root surface with 3 different restorative materials (scan area: 100 x 100 μm) (n = 5 for each restorative tested). The log [amide I:HPO_4^{2-}] for glass-ionomer cement was lower than that of the other 2 materials (P = 0.04). The statistical analysis of the restorative materials (v1) was: Type III sum of squares = 0.90, df = 2, mean square = 0.45, F = 4.19, and sig. = 0.03. The statistical analysis of sound and demineralized tissue (v2) was: Type III sum of squares = 0.51, df = 1, mean square = 0.51, F = 4.69, and sig. = 0.03. The statistical analysis of v1*v3 was: Type III sum of squares = 0.07, df = 2, mean square = 0.03, F = 0.32, and sig. = 0.73.

Scanning Electron Microscopy

After 3 weeks' culture of the mixed-species biofilm, glass-ionomer cement was the only restorative material that showed an increase in log Ca:P at the root surface adjacent to the restoration (P < 0.01) (Fig. 1); however, this material did not significantly increase log Ca:P on the enamel side (P = 0.72). The log Ca:P tailed off at distances farther from the restoration-tooth interfaces, but the ratio was not significantly different among the restorative materials tested on the enamel side (P = 0.87) or on the root surface (P = 0.89).

Fourier Transform Infrared Spectroscopy

The characteristics of tooth tissue and the 3 restorative materials, including their main chemical groups and FTIR spectra before and after 3 weeks' culture with a mixed-species biofilm, are shown in Fig. 2. Polymerization of liquid dimethacrylate monomers in the resin-modified glass-ionomer cement was light- and catalyst-initiated to provide a hard solid polymer; hence, the intensity of the carbonyl (C=O) band became very strong after 3 wks. Due to the different chemical compounds in the restorative materials tested, we did not attempt to identify an FTIR peak for comparison. Analysis of the overall FTIR spectral changes of the different restorative materials revealed that transmittance and peak area intensity of the glass-ionomer cement decreased after incubation in the artificial mouth. Transmittance and peak area intensity of the resin-modified glass-ionomer cement also decreased after incubation, although to a lesser extent. Resin composite was not affected after incubation with the biofilm.

Sound tissue in restored root surfaces had significantly lower log [amide I: HPO_4^{2-}] than demineralized tissue (Fig. 3). The FTIR spectrum of the root surface and enamel of control sound areas showed that the amide peaks were higher on the root surface than on enamel. The ANOVA results showed that 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 (Smales *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 liberation. 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 protolytic 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 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 HPO_4^{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 caries 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 biofilm generated in an artificial mouth—was thus rejected. The second null hypothesis—that restorative materials confer the same therapeutic effects on the enamel and root surface—is also rejected.

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