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Biodegradation of Resin-Dentin Interfaces Increases Bacterial Microleakage

ABSTRACT

Bis-GMA-containing resin composites and adhesives undergo biodegradation by human-saliva-derived esterases, yielding Bis-hydroxy-propoxy-phenyl-propane (Bis-HPPP). The hypothesis of this study is that the exposure of dental restorations to saliva-like esterase activities accelerates marginal bacterial microleakage. Resin composites (Scotchbond, Z250, 3M) bonded to human dentin were incubated in either buffer or dual-esterase media (pseudocholinesterase/cholesterol-esterase; PCE+CE), with activity levels simulating those of human saliva, for up to 90 days. Incubation solutions were analyzed for Bis-HPPP by high-performance liquid chromatography. Post-incubation, specimens were suspended in a chemostat-based biofilm fermentor cultivating *Streptococcus mutans* NG8, a primary species associated with dental caries, for 7 days. Bacterial microleakage was assessed by confocal laser scanning microscopy. Bis-HPPP production and depth and spatial volume of bacterial cell penetration within the interface increased with incubation time and were higher for 30- and 90-day PCE+CE *vs*. buffer-incubated groups, suggesting that biodegradation can contribute to the formation of recurrent decay.

KEY WORDS: biodegradation, bacterial microleakage, resin-dentin interface.

INTRODUCTION

Resin-composite matrices based on 2,2-Bis[4-(2-hydroxy-3-methacryloyloxy-propoxy)phenyl]propane (Bis-GMA) undergo significant chemical biodegradation when challenged by esterase activities (Jaffer *et al*., 2002; Finer and Santerre, 2004a) contained within human saliva (Lin *et al*., 2005). Hydrolytic cleavage of unhindered ester bonds at both ends of a Bis-GMA unit results in chemical breakdown, releasing bis-hydroxy-propoxy-phenyl-propane (Bis-HPPP), a marker of resin-matrix breakdown (Finer and Santerre, 2003). However, much of the current knowledge of resin biodegradation stems from observations of external surfaces of composite restorations interfacing with fluids from the oral cavity or simulated aging solutions (Santerre *et al*., 2001).

Bacterial microleakage is the most frequently cited post-operative complication among dentin-bonded composite restorations (Murray *et al*., 2002), and secondary caries is the principal cause of failure (Hickel and Manhart, 2001; Hicks *et al.,* 2003). *In vivo* biodegradation has been suggested as a potential contributor to secondary loss of adhesion, microleakage, and caries (Hickel and Manhart, 2001; Hashimoto *et al*., 2003; Donmez *et al*., 2005). Therefore, the physical and chemical integrity of a composite restoration's adhesive bond layer—the interface between the restoration and the tooth—is the most significant factor determining long-term clinical restoration success (Donmez *et al*., 2005). Very little research has focused on the impact of biodegradation along the tooth-resin interface. Of particular concern are proximal and cervical restorations where materials come into contact with wet dentinal substrate (Bouillaguet, 2004). Our hypothesis was that exposure of resin-composite restorations to saliva-like esterase activities accelerates marginal bacterial microleakage.

MATERIALS & METHODS

Preparation of Resin-Dentin Specimens

Dentin blocks cut from fully intact sterilized human third molars (University of Toronto Human Ethics protocol #15482) were bonded (Scotchbond MP, 3M, St. Paul, MN, USA) to composite resin (Z250, 3M) under sterile conditions, according to the manufacturer's instructions. We used a low-speed water-cooled rotary saw with a thin wafering blade (Isomet, Buehler, Lake Bluff, IL, USA) to prepare standardized (3 x 3 x 6 mm) resin-dentin specimens with cross-sectional areas of 3 mm^2. All regions of exposed dentin directly adjacent to the marginal interface were sealed with nail varnish to

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prevent access to the resin-dentin interface through cut dentinal tubules.

Degradation Media Incubation of Resin-Dentin Specimens

Specimens were randomly assigned to the following sterile incubation conditions: 7-, 30-, or 90-day incubation in phosphate buffer solution (PBS) or esterase solution (PCE+CE). We prepared PCE+CE by dissolving cholesterol esterase (CE) (Genzyme, Cambridge, MA, USA) and pseudocholinesterase (PCE) (Sigma, St. Louis, MO, USA) in PBS (Gibco, Grand Island, NY, USA) to match relevant esterase levels in human saliva (Finer and Santerre, 2004a; Lin *et al*., 2005). The media were replaced every 48 hrs. Media from defined incubation periods were pooled for high-performance liquid chromatography (HPLC) analysis.

Bis-HPPP By-product Isolation

We used a Waters™ HPLC system to isolate and quantify Bis-HPPP (Lin *et al*., 2005). Product identification was confirmed by mass spectrometry (QStar-XL, Applied Biosystems/MDS Sciex, Foster City, CA, USA).

Incubation of Resin-Dentin Specimens in Chemostat-based Biofilm Fermentor (CBBF)

Following assigned incubation periods, specimens were suspended within a closed-system biofilm fermentor designed to cultivate steady-state monoclonal biofilms of *Streptococcus mutans* NG8 over interfacial margins (Cvitkovitch *et al*., 2003). Fresh medium (Todd Hewitt yeast extract supplemented with 10 mM sucrose and 0.01% hog gastric mucin, 4X diluted) was pumped into the vessel at a flow rate of 0.72 L/day at a dilution rate of D = 0.075/hr, mimicking the resting flow rate of human saliva (Pratten *et al*., 2000). Daily maintenance of the CBBF included optical density readings, viable cell count, and pH adjustments. Specimens were aseptically removed after 7 days, rinsed with sterile water, and stained by means of a Live/Dead Baclight Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA).

Confocal Laser Scanning Microscopy (CLSM) Analysis

Stained specimens were assessed individually for bacterial penetration by CLSM (Zeiss LSM 510 META NLO, Carl Zeiss MicroImaging Inc., Toronto, ON, Canada). Six equidistant Z-stack series were captured along one side of each resin-dentin interface through a C-Apochromat 63x/1.2W (water-immersion) objective lens, zoom 2X. All 6 regions of interest (ROI) were standardized for orientation by the positioning of each specimen beneath the objective such that the composite-resin region was located at the top of the image screen, while the bottom of the image screen aligned with the dentinal region; the marginal interface was defined as that which occurred between the composite-resin and dentinal regions. CLSM Z-stack images were processed (ImageJ software) (Hope *et al*., 2002) to remove background fluorescence and allow for quantification of cells.

Statistical Analysis

We performed two-way ANOVA and conducted Scheffé's *post hoc* analysis ($p < 0.05$) to determine the effects of incubation time and condition on the amount of Bis-HPPP and total levels and depths of bacterial cells within the resin-dentin interface. All study groups were run in parallel, with 3 independent samples in each group. Each experiment was conducted 3 separate times.

RESULTS

Biodegradation

Levels of Bis-HPPP released from specimens incubated in PCE+CE media were significantly higher ($p < 0.0005$) than those from PBS-incubated specimens for all incubation times (Fig. 1A), with highest amounts measured at 90 days (297 ± 62 $\mu g/cm^2$). The highest rate of Bis-HPPP daily production occurred within the first 7 days (Fig. 1B). In comparison, total Bis-HPPP accumulation for the 90-day PBS-incubated specimens reached 9.68 ± 0.55 $\mu g/cm^2$, with no Bis-HPPP detected prior to 30 days (Fig. 1B).

Bacterial Microleakage

Specimens incubated for 90 days demonstrated significantly higher levels of interfacial cellular penetration ($p < 0.001$) than those incubated under the same culture conditions for shorter periods of time (Fig. 2A). Furthermore, after the 30-day incubation timepoint, the cumulative numbers of bacterial cells found penetrating the marginal interface were found to be significantly higher among PCE+CE-incubated specimens ($p < 0.005$) as compared with PBS-incubated specimens (Fig. 2A).

Within the 90-day incubation condition, specimens in PCE+CE demonstrated nearly 4 times as many ($p < 0.005$) bacteria than those in the PBS condition (Fig. 2B compared with 2C). Maximum interfacial depths of penetration were also nearly 4 times deeper among PCE+CE-incubated specimens than in their PBS counterparts ($p < 0.05$) (Fig. 2B compared with 2C).

Selected CLSM Z-stack images of samples are shown in Fig. 3. Among all 7-day-incubated specimens incubated in PBS or PCE+CE media, resin-dentin interfacial structural morphology resembled that of controls, which had been either non-incubated (3A) or incubated in either PBS or PCE+CE media, but not suspended within the CBBF (Figs. 3B, 3C; data for inoculated controls not shown). Limited amounts of adherence and penetration of *S. mutans* biofilm cells were observed among interfacial surface micro-porosities for 7-day-incubated specimens, up to a maximum depth of 4 μm (Figs. 2B, 3B). All component layers of the resin-dentin margin appeared linearly oriented to one another, and well-infiltrated by the priming resin. In 30-day samples, morphological changes to the resin-dentin interfacial margin emerged, albeit to various extents, depending on the incubation medium (Figs. 3D, 3E). For PBS samples, dentinal tubules of the hybrid layer commonly experienced finite structural degradation, particularly near the composite resin-resin

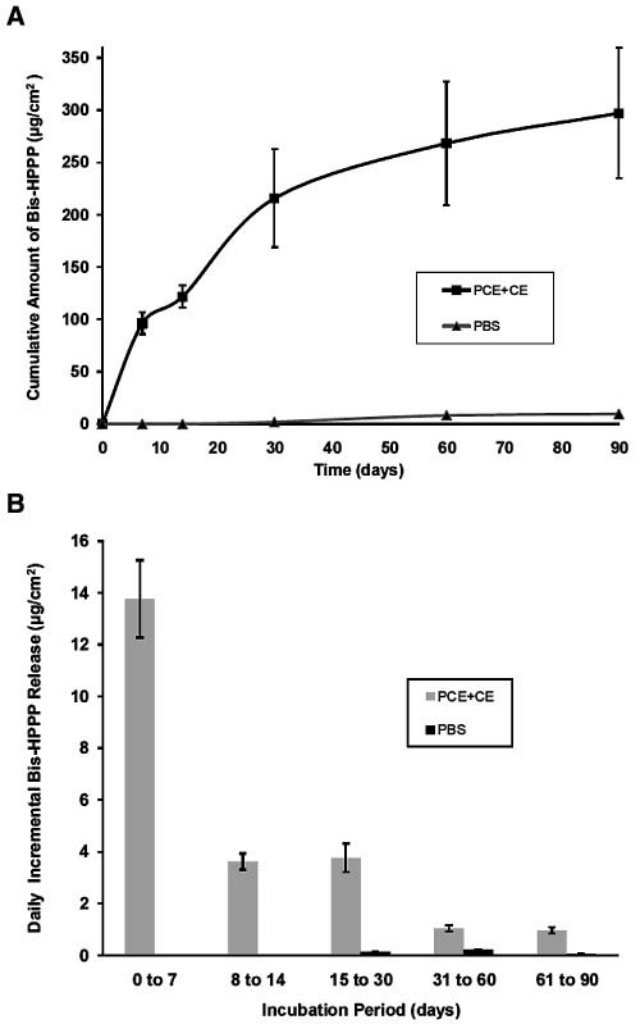

Figure 1. Bis-HPPP release from resin-dentin specimens. **(A)** Cumulative amount of Bis-HPPP produced from resin-dentin specimens incubated in PCE+CE or PBS buffer for 7, 14, 30, and 90 days (pH 7, 37°C). **(B)** Incremental amount of Bis-HPPP produced from resin-dentin specimens incubated in PCE+CE or PBS buffer. All data are reported with standard error of the mean (n = 3).

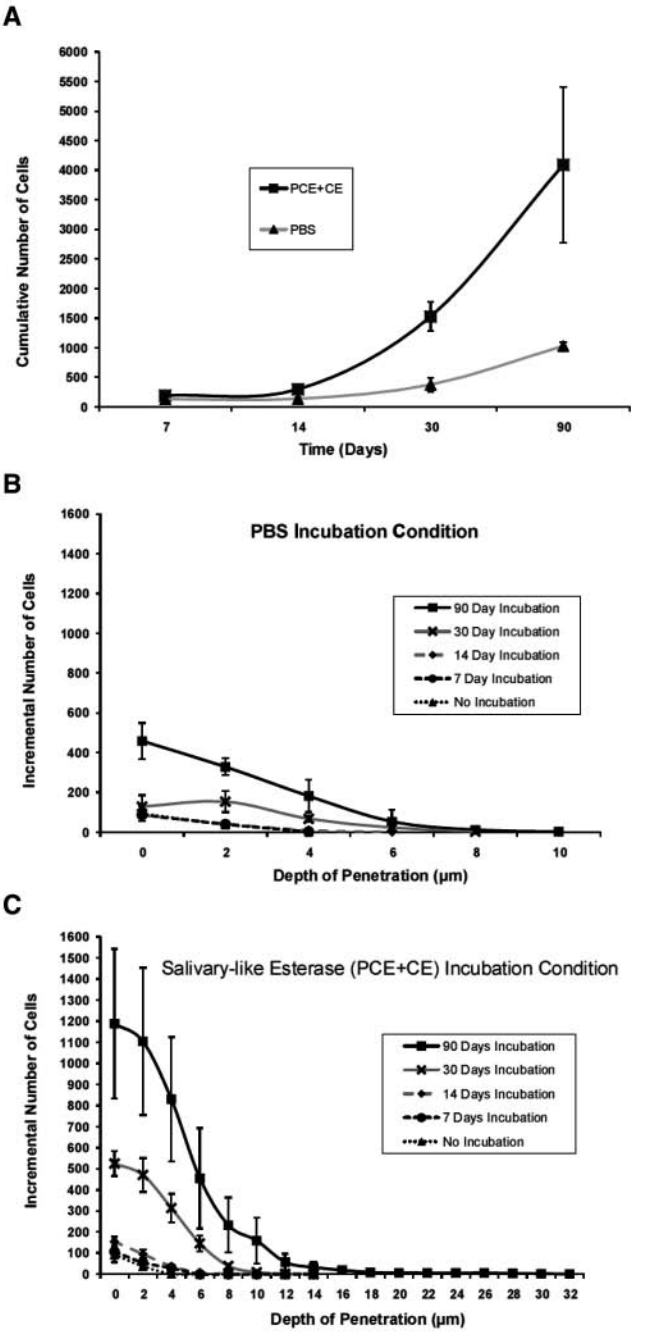

Figure 2. Bacterial penetration along the resin-dentin marginal interface. **(A)** Cumulative numbers of bacterial cells found penetrating the marginal interface for PBS controls and PCE+CE-incubated specimens over time. Number of cells *vs.* depth of penetration at interfacial ROIs of **(B)** PBS-incubated and **(C)** PCE+CE-incubated specimens for 0, 7, 14, 30, and 90 days (pH 7, 3°C). All data are reported with standard error of the mean (n = 3).

adhesive region of the marginal interface. These changes were more extensive for the PCE+CE specimens (compare Figs. 3D and 3E).

In PBS and PCE+CE specimens at 90 days, junctions among the resin restorations, the adhesive layer, and the hybrid layer were interrupted and with an undulating pattern (Figs. 3F, 3G).

Two of the numerous examples of gross interface deformation found in the PCE+CE samples are shown in Fig. 4. A distinctive interfacial void at the top of the hybrid layer, where the *S. mutans* biofilm adhered to the top and bottom of the axial walls, is shown in Fig. 4A. At sample depths of 6 µm and over, biofilm growth extended to span the entire width of the void. An interfacial gap spanning more than 20 µm is shown in Fig. 4B. Sagittal sections through this particular ROI reveal a characteristically distinct three-dimensional mushroom-shaped pattern of *S. mutans* biofilm growth (Hope *et al*., 2002) extending up to 34 µm beneath the surface of the interface (images not shown).

DISCUSSION

Relative to PBS controls, PCE+CE-incubated resin-dentin specimens generated significantly higher amounts of Bis-HPPP ($p < 0.0001$) at all incubation timepoints and resulted in greater bacterial surface adherence and penetration along the resin-dentin

marginal interface with time. Therefore, the hypothesis that exposure of resin-composite restorations to saliva-like esterase activities accelerates marginal bacterial microleakage was shown to be true. Analysis of the current data demonstrated that biodegradation of resin-adhesives and composites is a time-dependent process that progressively compromised the clinical value of resin materials with increased incubation time.

The degradation by-product of choice for this study, Bis-HPPP, is a derivative of Bis-GMA, a common monomer used in both adhesive and composite materials investigated in the current study. Bis-HPPP production was shown to be a good indicator of overall resin matrix degradation (Finer and Santerre, 2007; Shokati *et al*., 2010).

Periodic daily incremental release rates of Bis-HPPP within the 90-day period for PCE+CE-incubated specimens varied over time. The highest rate of accumulation occurred within the first 7 days of PCE+CE incubation (13.8 ± 1.49 μm/cm^2 *per* day), decreased by a factor of 4 between 8 and 14 days of PCE+CE incubation, and reached a plateau at a constant release rate of 1 ± 0.1 μm/cm^2 *per* day between days 30 and 90. Theoretically, for the first 7 days of incubation, hydrolytic reaction rates are defined primarily by the catalytic activity of the esterases. By the 8th day of incubation, the number of readily accessible ester linkages within the Bis-GMA-based resin matrix gradually declines. The rate-controlling factor becomes the physical access of esterases to the un-reacted ester substrate, slowing the hydrolytic process. After 30 days of incubation, the most readily accessible ester linkages within the resin matrix have become hydrolyzed. At this point, longer-term biochemical breakdown of the resin matrix depends on rates at which previously inaccessible ester linkages become unmasked by the elution of degraded oligomers (Finer and Santerre, 2004b, 2007).

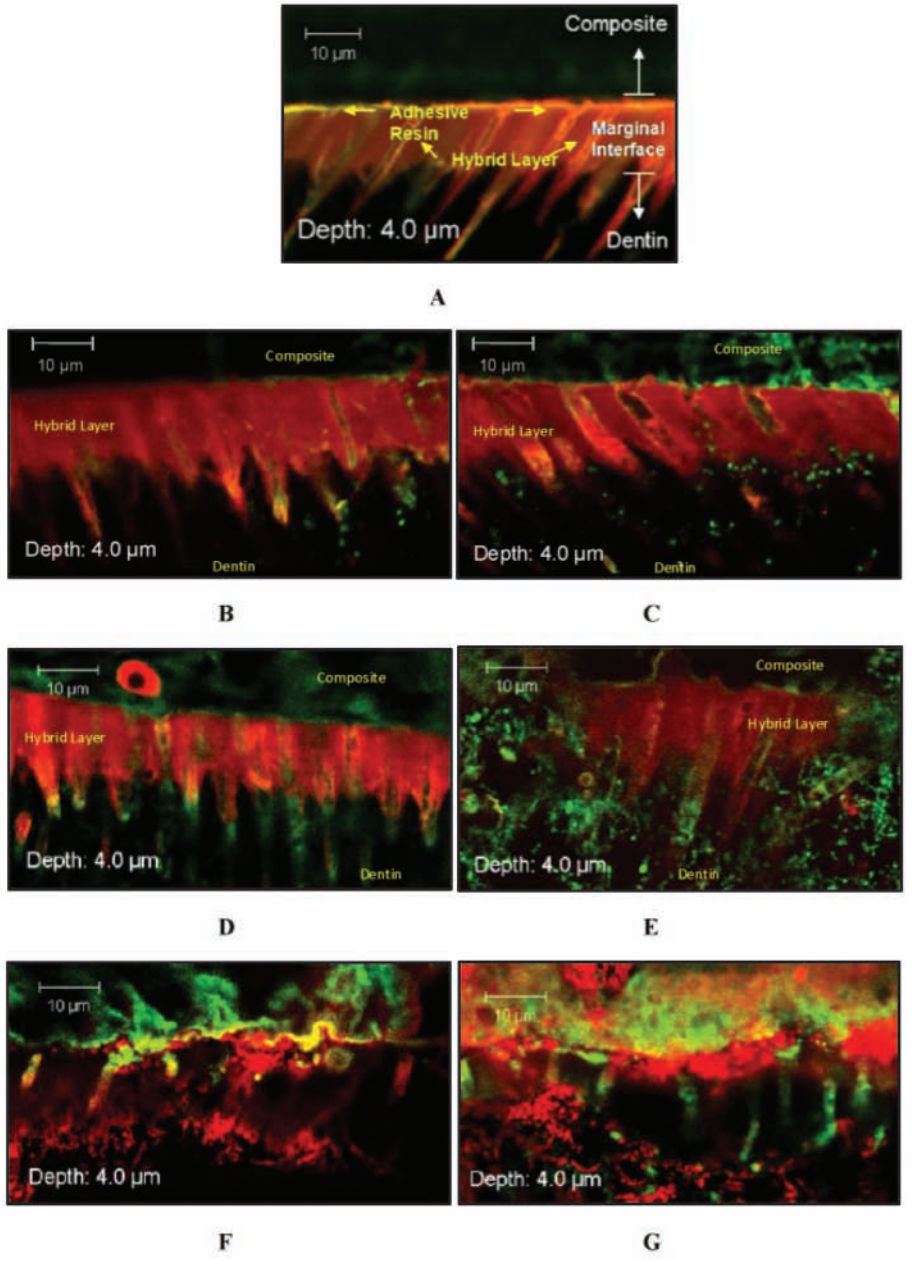

Figure 3. Selected *Z*-stack image series captured from interfacial margins of resin-dentin specimens assigned to either **(A)** non-incubated, **(B)** 7-day PBS incubation, **(C)** 7-day PCE+CE incubation, **(D)** 30-day PBS incubation, **(E)** 30-day PCE+CE incubation, **(F)** 90-day PBS incubation, or **(G)** 90-day PCE+CE incubation. Interfacial zones (composite, adhesive, hybrid layer, and dentin) are distinguishable in A and, to a lesser extent, in B-E; however, in F and G, the organization of these marginal components is disrupted. Resin impregnation of dentinal tubules in the hybrid layer is disrupted (F and G). Specimens were stained by means of a Live/Dead Baclight Viability Kit (magnification X62, 2X zoom). Live cells indicated by green fluorescence through interaction with Syto9; dead cells indicated by red fluorescence through interaction with propidium iodide.

Bacterial adherence and penetration among the superficial sub-layers (0-4 μm) of minimally compromised interfacial margins were mainly localized to the top and bottom of the hybrid layer in samples incubated for 7 days with either PBS or PCE+CE. Intrinsic interfacial porosities are often formed during bond application, potentially generated by polymerization shrinkage at the top of the hybrid layer, or incomplete resin impregnation of demineralization dentin occurs at the bottom (Suppa *et al.*, 2005). Both phenomena may also account for the

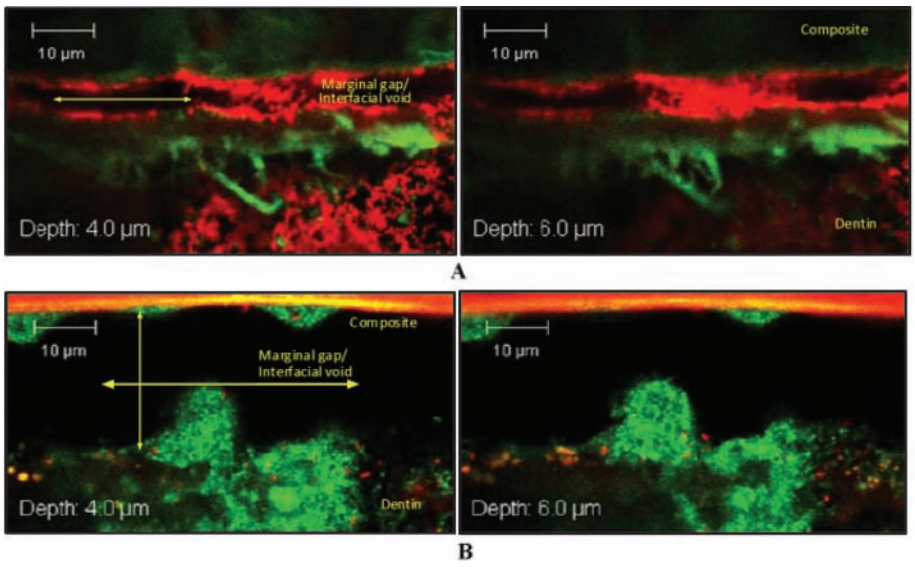

Figure 4. Selected *Z*-stack image series captured at interfacial ROIs of 2 90-day PCE+CE-incubated resin-dentin specimens. **(A)** Interfacial void spanning approximately 4-5 µm in height. **(B)** Interfacial void spanning over 20 µm in height. Characteristic of three-dimensional biofilm growth are interstitial voids that can be seen among fluorescently stained *S. mutans* microcolonies. In (B), large mushroom-shaped biofilm structures are found colonizing both the top and bottom axial walls. Specimens were stained by means of a Live/Dead Baclight Viability Kit (magnification X62, 2X zoom). Live cells indicated by green fluorescence through interaction with Syto9; dead cells indicated by red fluorescence through interaction with propidium iodide.

superficial bacterial microleakage observed at the interfacial sublayers of control resin-dentin specimens un-incubated with degradation media (data not shown).

Discrepancy between the depth of the demineralization and resin infiltration is common among commercial three-step "etch-and-rinse" adhesives such as Scotch Bond Multi-Purpose (Spencer and Wang, 2002). Oral streptococcal cells span approximately 0.5 to 0.7 µm in diameter (Love and Jenkinson, 2002). Given access, they can penetrate nanometer-sized voids at the bottom of the hybrid layer and directly bind to intra-tubular collagen type I components of the dentinal tubules (Love and Jenkinson, 2002). Furthermore, given the widely reported effects of water sorption at the top and bottom of the restorative interface (Sauro *et al.*, 2009), as well as associated elution of adhesive resin components over time in both *in vivo* (Pashley *et al.*, 2004) and *in vitro* conditions (Hebling *et al*., 2005; Shokati *et al*., 2010), nanometer-sized interfacial voids can expand with prolonged incubation in media (Suppa *et al.*, 2005). The results from this investigation corroborated such findings, since localization of bacterial microleakage among resin-dentin specimens incubated for 7- and 30-day time periods was centralized near the top and base of the hybrid layer. In light of significant interfacial disruptions found among specimens incubated for 90 days, though, bacterial microleakage for all samples at this time period was non-specific and occurred across the entire interfacial span.

Qualitative assessments of interfacial structural integrity among resin-dentin specimens also led to the conclusion that while incubation in PBS altered the morphology of the marginal interface over time (Pashley *et al*., 2004), exposure to salivary-like esterase activities of PCE+CE media greatly amplified the intrinsic effects of hydrolytic processes. The reduction of signal scatter in the inter-tubular layer of the hybrid zone suggests a loss of resin and/or mineral content, a morphological change consistent with that of carious dentin (Zavgorodniy *et al*., 2008). While an overall reduction in red fluorescence signal scatter was observed at inter-tubular regions of the hybrid layer in 90-day PBS-incubated specimens, it was almost entirely absent among those of 90-day PCE+CE-incubated specimens.

The presence of blister-like voids and the observed undulating pattern of the interface layer may be a consequence of interfacial water sorption (Sauro *et al*., 2008) causing swelling and plasticization of resin polymers (Ferracane, 2006). Given that ongoing hydrolytic processes can propagate marginal gap formation over time (Hashimoto *et al*., 2003), it is then not surprising that the largest marginal gaps were found exclusively among 90-day PCE+CE-incubated specimens, which generated the greatest amounts of Bis-HPPP.

It was also within the expanded marginal gap region of 90-day PCE+CE-incubated specimens that the most extensive colonization of *S. mutans* biofilms was found. Highly characteristic biofilm structures (Lewandowski *et al*., 2007) were anchored to the composite resin or dentinal axial walls of marginal gaps spanning 10 µm or more. Recently, it was suggested that larger-sized marginal gaps provide the necessary space and access to the nutrients necessary for successful colonization by larger numbers of micro-organisms (Totiam *et al*., 2007).

It is the current belief that secondary loss of marginal integrity is primarily attributed to mechanical forces such as occlusal loading (Bouillaguet, 2004) as well as thermal stress and polymerization contraction (van Noort, 1994). Yet, analysis of the increasingly emerging data in the scientific literature has suggested a potential for secondary loss of adhesion due to *in vivo* chemical attack (Hickel and Manhart, 2001; Hashimoto *et al*., 2003; Donmez *et al*., 2005). The current findings make this case abundantly clear. Evidence of increased bacterial penetration coupled with dentin demineralization suggests that the biodegradation process can contribute to the formation of recurrent decay—the most common cause of restoration failure (Hicks *et al*., 2003).

The results of this study also demonstrated high reproducibility as well as clinical relevance, a factor which is imperative in the evaluation of biomaterials with an *in vitro* experimental

system. As a result, this model shows great potential for further development into a standardized testing system of biochemical stability among various commercial adhesive and composite materials prior to use in clinical settings. Where hybrid layer interruption and marginal gaps do occur, the current system presents a practical non-invasive imaging method for intact biofilms adhering to and proliferating on and within the resin-dentin interface. To the best of our knowledge, the current investigation provides the first physiologically relevant *in vitro* characterization of bacterial microleakage within the resin-dentin interface.

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