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On the Fatigue Behavior of Resin-Dentin Bonds after Degradation by Biofilm

Mustafa Murat Mutluay^{1,2}, Ke Zhang³, Heonjune Ryou², Mobin Yahyazadefar², Hessam Majd², Hockin H. K. Xu³, and Dwayne Arola^{2,3,*}

¹Adhesive Dentistry Research Group, Institute of Dentistry, University of Turku, Turku, Finland

²Department of Mechanical Engineering, University of Maryland Baltimore County, Baltimore, MD, USA ³Department of Endodontics, Prosthodontics, and Operative Dentistry, Dental School, University of Maryland Baltimore, Baltimore, MD USA

Abstract

The durability of resin-dentin bonds is a growing concern in the placement of composite restorations. Most reported evaluations concerning the mechanical behavior of the bonded interface are conducted using static loading to failure only. They also do not account for the acid production of biofilms, which is one of the most common contributors to interfacial failures *in vivo*. In this investigation resin-dentin bonded interface specimens were exposed to *S. mutans* for 14 days and then subjected to quasi-static or cyclic four-point flexure to failure. Control specimens (without biofilm) were evaluated after aging for one and fourteen days. While no significant difference in flexure strength resulted from the duration of water aging (66.2 MPa vs 56.9 MPa), biofilm exposure caused a significant reduction in strength (29.3 MPa; $p < 0.000$). After water aging for one and fourteen days the apparent endurance limits were 13.0 MPa and 13.1 MPa, respectively. Biofilm treatment caused a significant ($p < 0.001$) reduction in fatigue resistance of the interface, and the endurance limit was reduced to 9.9 MPa. Fatigue failure of the control specimens initiated within the resin composite adjacent to the interface, whereas failure of the biofilm treated specimens initiated within the hybrid layer and appeared attributed to the localized demineralization of dentin. Biofilm degradation is an important consideration in assessing the durability of resin-dentin bonds.

Keywords

Biofilm; Bonding; Durability; Fatigue; Hybrid Layer; Resin Composite

INTRODUCTION

Resin composites are now the primary material for tooth cavity restorations [Ferracane, 2011]. In the United States, over 45% of all restorations placed in 2005 were resin

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***Corresponding Author:** Dwayne D. Arola, Ph.D., Department of Mechanical Engineering, University of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore, MD 21250 USA, darola@umbc.edu, (410) 455-3310 (v), (410) 455-1052 (f).

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composites, with the remainder consisting of roughly 32% amalgam and 22% crowns [Beazoglou et al., 2007]. But there is growing concern that bonded composite restorations have higher failure rates [e.g. Bernardo et al., 2007; Demarco et al., 2012]. In a survey of nearly 3,000 restorations placed in adult patients, over 50% consisted of replacements [Sunnegårdh-Grönberg et al., 2009]; the average service life of the composites was nearly one third that of amalgam (6 years vs 16 years, respectively). Secondary caries, degradation of the restorative margins and fracture of the restoration are the primary modes of composite restoration failure, with secondary caries being the most common overall [Deligeorgi et al., 2001; Brunthaler et al., 2003].

Composite restorations are bonded to tooth structure and their success is dependent on the strength of adhesion. Consequently, bond strength testing has been used to quantify the adhesion of restorative materials to dentin and enamel since the invention of acid etching [Buonocore, 1955]. Micro-tensile and shear tests are the primary approaches for evaluating the qualities of new materials and bonding procedures [Pashley et al., 1999; De Munck et al., 2012]. In their application, bond strength has been adopted as the metric for indicating performance in the oral environment, and high strength is considered indicative of longevity. However, there are concerns regarding the applicability of these testing methods to understanding clinical failures [Van Meerbeek et al., 2003; Roulet, 2012; Söderholm, 2012]. In fact, a recent meeting of the Academy of Dental Materials was focused on their role (and potential shortcomings) in adhesive dentistry [Ferracane et al., 2010]. Perhaps the prevailing concern is that the results of *in vitro* experiments do not reflect the reality of failures *in vivo*, and there is little correlation to clinical behavior [Ferracane, 2012].

For composite restorations to achieve longevity, both the composite material and the adhesive bonds to tooth structure must resist damage over many years of function. Indeed, cyclic loading is used in the evaluation of composite restoratives [e.g. Braem et al., 1994; Baran et al., 2001; Drummond, 2008; Drummond et al., 2009; Shah et al., 2009]. Cyclic stresses transmitted across the bonded interface may cause degradation over time [Spencer et al., 2010; Pashley et al., 2011]. However, in comparison to the efforts placed in evaluating the interface using microtensile tests, fatigue degradation of resin-dentin [Drummond et al., 1996; Frankeberger et al., 2003; Soappman et al., 2007; Staninec et al., 2008; Belli et al., 2010] and resin-enamel [De Munck et al., 2005; Erickson et al., 2006; 2008; 2009a; Barkmeier et al., 2009] interfaces has received very limited attention.

Equally important to fatigue of the bonded interface, resin composites accumulate more biofilm/plaque than other restorative materials [Beyth et al., 2007]. Degradation of restorative materials caused by biofilm has been investigated [e.g. Beyth et al., 2008; Fúcio et al., 2008; Busscher et al., 2010]. Biofilms cause an increase in the surface roughness of resin composites, but surprisingly little or no changes in hardness [Beyth et al., 2008]. Furthermore, the surface topography is important to the adhesive strength of bacteria [Emerson et al., 2006] and an increase in surface roughness encourages biofilm formation [Busscher et al., 2010]. Though surface characteristics are clearly important, they do not address interface durability. Microleakage permitted by interfacial degradation or fatigue-related damage could enable bacteria to invade the interface and accelerate failures. However, there has been no report on the degradation of dentin-resin bonds subjected to a combination of biofilm challenge and cyclic loading.

The primary objectives of this study were to, for this first time 1) quantify the durability of resin-dentin interfaces involving commercial resin composite after exposure to a biofilm of *S. mutans*, and 2) to distinguish the primary mechanisms contributing to bond failure under cyclic loading. It was hypothesized that biofilm exposure would cause a significant reduction in strength of the bonded interface under both static and cyclic loading.

EXPERIMENTAL MATERIALS AND METHODS

A novel specimen geometry consisting of twin bonded resin-dentin interfaces was developed for this study (Figure 1). The bonded interface specimens were prepared with the coronal dentin of caries-free human third molars. All extracted teeth were obtained with record of age (18 age 30) and gender from participating clinics in Maryland according to a protocol approved by the University of Maryland Baltimore County (#Y04DA23151). The teeth were stored in Hank's Balanced Salt Solution (HBSS) for less than 1 month, and then sectioned using a computer-controlled grinder (Chevalier Smart-H818II, Chevalier Machinery, Santa Fe Springs, CA, USA) with diamond abrasive slicing wheels (#320 mesh abrasives) and water spray coolant. Rectangular beams of mid-coronal dentin with cross-section of $2 \times 2 \times 12$ mm³ were sectioned from the mid-coronal region as shown in Figure 1(a). Primer and adhesive (Clearfil SE Bond, Lot 062127, Kuraray America, Houston, TX, USA) were applied according to the manufacturer's recommendations to the two opposing surfaces with lumens parallel to the section surface. The dentin beams were placed in a specialized mold for bonding such that the lumens were oriented parallel to the interface (Figure 1(b)). Restorative resin composite (Clearfil AP-X, A2 color, Lot 1136AA; Kuraray America) was applied incrementally according to the manufacturer's recommendations from the dentin beam surface as necessary to fill the two mold cavities. The composite was cured on both sides for 40 seconds using a quartz-tungsten-halogen light-curing unit (Demetron VCL 401, Demetron, CA, USA) with output intensity of 600 mW/cm² and with tip diameter wider than 10 mm. The molded sections selected for biofilm exposure were rinsed under distilled water and then stored at 4°C in water containing 0.05% thymol until required (Cheng et al., 2008).

The molded sections were released from the mold and then aged (controls) or treated by exposure to *Streptococcus mutans* (*S. mutans*) bacteria (ATCC 700610, UA159, American Type Culture, Manassas, VA) according to a protocol approved by the University of Maryland Baltimore. *S. mutans* was chosen because it is a cariogenic, aerotolerant anaerobic bacterium and the primary causative agent of dental caries [Loesche, 1986]. Each molded specimen receiving biofilm treatment was placed in a well of a 24-well plate, inoculated with 1.5 mL of the inoculation medium, and incubated at 5% CO₂ and 37°C for 14 days to simulate the demineralized caries-affected dentin (Azevedo et al., 2011; de Carvalho et al., 2008; Markezan et al., 2009; Meyer-Lueckel et al., 2006). To prepare the inoculation medium, a 15 µL quantity of stock bacteria was added to 15 mL of growth medium and incubated at 37°C with 5% CO₂ for 16 hrs (Cheng et al., 2012; Exterkate et al., 2010). The "growth medium" consisted of a Brain Heart Infusion (BHI) broth (BD, Franklin Lakes, NJ) supplemented with 0.2% sucrose. The BHI broth consists of a mixture of 6.0 g brain heart (infusion from solids), 6.0 g peptic digest of animal tissue, 5.0 g Sodium chloride, 3.0 g dextrose, 14.5 g pancreatic digest of gelatin and 2.5 g disodium phosphate per liter of purified water. During this culture the *S. mutans* were suspended in the BHI broth.

A live/dead assay was performed to ensure that the *S. mutans* were successfully incubated onto the surfaces of the specimens. Here, the biofilms that developed on a molded section of the interface after 3 days' inoculation were washed with phosphate buffered saline (PBS) and stained using the BacLight live/dead bacterial viability kit (Molecular Probes, Eugene, OR). Live bacteria were stained with Syto 9 to produce green fluorescence, while compromised bacteria were stained with propidium iodide to produce a red fluorescence (Cheng et al., 2012). The biofilm was then examined using an epifluorescence microscope (Eclipse TE2000-S, Nikon, Melville, NY). Photo micrographs of the biofilms and results after live/dead staining are shown in Figure 2(a) and 2(b), respectively. Live bacteria on the surface are stained green and dead bacteria are stained red (Figure 2(b)). Regions with orange or yellow colors represent areas where live and dead bacteria were close to, or on the

top of, each other. As evident in Figure 2, the bacteria were successfully incubated and alive on the molded sections with interface, with uniform coverage over the surface.

Over the 14 day exposure period the growth medium was changed every 24 hours, by transferring the disks to a new 24-well plate with fresh growth medium. Control specimens of the bonded interface (without biofilm) were stored in deionized water at room temperature (22° C) for either one day (i.e. 24 hours) or 14 days. After completing the duration of exposure, sectioning was performed using the grinder to obtain two interface specimens from each molded section with final geometry of 2×2×12 mm³. Based on the storage and sectioning process used to obtain both the biofilm treated and control groups, each specimen had two opposing exposed surfaces, and two that were freshly sectioned (i.e. without direct exposure to biofilm).

Quasi-static and cyclic four-point flexure loading of the specimens was performed using a universal testing system (EnduraTEC Model ELF 3200, Minnetonka, MN, USA) with load capacity and sensitivity of 225 N and ±0.01 N, respectively. Quasi-static loading was performed according to Figure 1(c) with the specimens maintained in water at room temperature using displacement control feedback at a crosshead rate of 0.06 mm/min after Arola et al [2005]. The instantaneous load and load-line displacement were monitored throughout loading at a frequency of 4 Hz. The flexural strength of the beams was determined using conventional beam theory [Popov, 1978] in terms of the maximum applied load (P) and beam geometry (width, thickness h) according to $3Pl/bh^2$, where l is the distance from interior and exterior supports (l=3 mm). Ten specimens were evaluated after biofilm exposure and of both control groups (N=10) after water aging, for a total of 30 specimens. The flexure strengths were compared using a one-way ANOVA and Tukey's HSD post-hoc analysis with the critical value (alpha) set at 0.05.

Cyclic loading of the specimens was conducted with water bath hydration using the same configuration for the quasi-static evaluation (Figure 1(c)) under load control with frequency of 4 Hz and stress ratio (R = ratio of minimum to maximum cyclic load) of 0.1. The surfaces exposed to biofilm (or water) were always arranged to coincide with the maximum tensile and compressive stresses. Fatigue testing was initiated using a maximum cyclic stress of approximately 90% of the flexural strength identified from the quasi-static experiments. For successive specimens, the maximum cyclic load was decreased in increments ranging from 5 to 15 MPa according to the staircase method of evaluation [Collins, 1981]. The process continued until reaching the flexure stress amplitude at which specimens did not fracture within 1.2×10^6 cycles. The stress-life (S-N) fatigue distribution was evaluated by plotting the cyclic stress amplitude in terms of the number of cycles to failure. The fatigue life distribution of the specimens in each group that underwent fatigue failure was fit using non-linear regression with a Basquin-type model [Stephens et al., 2001]

$$\sigma = A(N)^B \quad (1)$$

where A and B are the fatigue-life coefficient and exponent, respectively. These constants were obtained from a power law regression of the fatigue responses plotted on a log-normal scale. The apparent endurance limit was estimated from the models for a fatigue limit defined at 1×10^7 cycles, which been used in previous evaluations [Arola and Reprogel, 2005; 2006]. Twenty specimens were evaluated after biofilm exposure, and of both control groups, for a overall total of 60 fatigue specimens. Results for each group were compared using the Wilcoxon Sum Rank Test with p 0.05 considered significant.

The flexure specimen geometry for this study was designed with twin interfaces such that each experiences equivalent bending moments and corresponding normal stresses. One of

the interfaces undergoes failure and the second interface, which has experienced the same stress history, effectively “freezes” the microstructure at that moment. Though differences in the population of defects can cause one interface to fail preferentially, the second remains unbroken and facilitates further evaluation of the preserved microstructure. Thus, both the fractured and unbroken interfaces were evaluated using a combination of techniques. All biofilm treated specimens were placed in an ultrasonic bath of distilled water for 15 minutes after cyclic loading to assist in removal of the biofilm.

The unbroken interfaces were evaluated using nanoscopic Dynamic Mechanical Analysis (nanoDMA). To perform nanoDMA evaluation, the portion of specimen containing the surviving resin-dentin interface was cold-mounted in Epofix epoxy resin (Struers, Cleveland, OH, USA). The side of the specimen was exposed, thereby revealing the tension and compression areas of the specimen. Polishing was performed with diamond particle suspensions (Buehler) of sizes 9, 3, and 0.04 μm to produce a highly polished surface with a roughness of less than 50 nm RMS. NanoDMA was performed with a Triboindenter (Model TI 900, Hysitron, Minneapolis, MN, USA) and a Berkovich diamond indenter with a 100-nm tip radius. Scanning mode dynamic loading was conducted over scan areas of 30 μm $\times$ 30 μm with 4 μN contact load, 2 μN dynamic loading amplitude and dynamic loading frequency of 100 Hz. The contact load and displacement signals were used to calculate the phase angle and to generate maps of the complex (E^*) modulus distribution for the dentin, resin adhesive and hybrid zone and restorative resin. Scanning was performed with hydration using a layer of ethylene glycol over the specimen surface to prevent water evaporation. An unloaded interface specimen (control) was also evaluated using these techniques and after immersion in water for 14 days. Additional details regarding application of nanoDMA in evaluating the resin-dentin interface is described in [Ryou et al., 2011; 2012]. Separate scans were made along the interface in the region of cyclic tension, near the neutral axis and in the region of cyclic compression.

The fracture surfaces and intact bonded interfaces of selected specimens were also inspected using a Scanning Electron Microscope (SEM: JEOL Model JSM-5600, Peabody MA, USA) in secondary electron imaging (SEI) mode. Prior to this analysis the specimens were dehydrated in an ascending ethanol series (70–100%), fixed in Hexamethyldisilazane, polished lightly using #600 mesh emory cloth and then sputtered with gold/palladium to enhance conductance of the dentin and resin adhesive. The fracture surfaces were inspected to distinguish differences between static and fatigue loading and to identify the origins of failure. The unbroken interfaces were inspected to identify if damage developed as a result of cyclic loading and the location.

NUMERICAL EVALUATION

Due to the presence of two interfaces, it was necessary to evaluate the stress distribution in the resin-dentin specimens. Using twin interfaces in a flexure loading configuration could appear to complicate the stress distribution, and a model serves to provide a clear understanding of the stresses acting on the interface. In addition, it was desired to use beam theory for estimating the stress distribution resulting from flexural loading, and a finite element model could be used to assess its applicability. Thus, a two-dimensional finite element analysis was performed using commercial software (ABAQUS 6.7-3; Dassault Systemes Americas Corp., Waltham, MA, USA). Though not required, a full model was developed for the beam to simulate the resulting stress and strain distribution. The model specimen was defined having three regions (Figure 3a), including the resin composite, resin adhesive and dentin, and meshed with approximately 3600 nodes and 1200 type CPE4 elements. For convenience, the materials were treated as linear elastic with elastic modulus (E) and Poisson's ratio (ν) defined for dentin ($E=15$ GPa, $\nu=0.29$) [37], resin composite (6.0

GPa, 0.26) [AP-X, Kuraray USA] and resin adhesive (4.4 GPa, 0.24) [38]. Due to the limited information for the macroscopic elastic modulus of resin-infiltrated dentin, the hybrid layer and resin adhesive were combined and considered to have consistent elastic properties (4.4 GPa, 0.24). It was identified using nanoDMA that the extent of sub-surface demineralization caused by the acid production of biofilm was limited. Therefore, it was not necessary to modify the model to account for changes in elastic moduli.

The model interface specimens were subjected to simulated flexural loading according to the experimental configuration in Figure 1(c). A portion of the beam is shown from the axis of symmetry to just beyond the interior contact point in Figure 3(a). The stainless steel loading pins used in the experiments were defined as rigid body shells that enabled the development of contact stresses at the beam surfaces. A flexural load of 10 Newtons was applied, which falls within the range in loads applied during fatigue testing and the resulting stress and strain distributions were evaluated. One expected criticism of the flexural loading arrangement is that it is not a “clinically relevant” mode of loading. Clearly flexure is not applied to bonded composite restorations *in vivo*. However, flexure is a mode of loading used herein to generate a stress state comprised of a gradient along the interface with largest stress at the exterior surfaces. Finite element evaluations of teeth restored with bonded resin composites show that the interfacial stress consists of a stress gradient as well [Arola et al., 2001; Asmussen and Peutzfeldt, 2008]. Nevertheless, the model interface specimens were also subjected to simulated uniaxial tension loading for completeness, and the stress distributions for tension and flexure were compared. To aid in this comparison, the magnitude of applied axial load was chosen to result in an equivalent maximum principal stress in the dentin for both flexure and tension.

Results from finite element analysis for flexure loading of the bonded resin-dentin specimens are shown in Figure 3. The normal strain (ϵ_x) and normal stress (σ_x) distributions within the specimen from the axis of symmetry are shown in Figure 3(b) and Figure 3(c), respectively. The largest strain develops within the hybrid layer and resin adhesive, and is nearly three times larger than that within the adjacent materials (Figure 3(b)). The stress distribution in Figure 3(c) shows that contact loading causes a concentration of stress on the compressive side of the beam, but that contact does not have a large influence on the stress distribution at the interface.

The maximum normal stress along the tensile surface of the specimen is plotted in Figure 4(a) from the axis of symmetry (center of dentin), across the adhesive interface and continuing in the composite. That distribution is compared with the stress predicted using beam theory for a homogeneous specimen with identical geometry and loading configuration. The tensile stress distribution is not influenced substantially by the contact stresses. The largest normal stress within the region of constant moment develops at the boundary of dentin and the hybrid layer, and is within 5% of that predicted using beam theory. Nevertheless, the normal stress is clearly not constant between the two loading pins and is substantially influenced by the adhesive interface. The stress distribution resulting from flexure (from Figure 4(a)) is compared with results from simulated uniaxial tensile loading of the interface specimen in Figure 4(b). An axial load of 45 Newtons was applied such that the maximum stresses caused by the two loading conditions are equivalent. In tension, the maximum stress develops in the dentin and there is a reduction in the normal stress across the hybrid and adhesive layers. As evident from this comparison, the stress distributions at the surface of the beam resulting from flexure and tension are essentially identical.

RESULTS

Results from quasi-static flexure loading of the control and treated interface specimens to failure are shown in Figure 5(a). The strength of the bonded interface exposed to biofilm (29.3 ± 11.1 MPa) was significantly lower ($p = 0.0000$) than that obtained for the two controls. Although the flexure strength after 14 day aging (56.9 ± 15.9 MPa) was lower than that after one day (66.2 ± 10.4 MPa), the difference was not significant ($p > 0.05$).

Fatigue life diagrams obtained from cyclic loading of the bonded interface specimens are shown in Figure 5(b). The constants (i.e. A, B) obtained from fitting the power law models to the three responses are also presented for comparison. It is important to note that the bonded interface exposed to biofilm exhibited the lowest fatigue strength over the entire fatigue life regime. When defined at 1×10^7 cycles, the apparent endurance limit for the interface after water aging for one and 14 days was 13.0 MPa and 13.1 MPa, respectively. For the specimens exposed to biofilm that value was nearly 25% lower (9.9 MPa). The fatigue life distributions for water aging one and 14 days were not significantly different ($Z = -0.08; p = 0.468$). However, after biofilm treatment the fatigue life was significantly lower than that after water aging ($Z = -3.11; p = 0.001$), regardless of the period of storage.

Representative fracture surfaces for specimens after water aging (1 day) and biofilm exposure are shown in Figure 6(a) and 6(b), respectively. The tensile surface is arranged at the top of the micrograph in both figures. Fatigue failure of the water-aged specimens initiated within the resin composite on the tensile side of the neutral axis, as distinguished by the fracture characteristics and compression shear lip (S) in Figure 6(a). In the control specimens the fracture surfaces were comprised predominately of resin composite and resin adhesive; none of the control specimen failures initiated within the dentin. In specimens exposed to biofilm, fatigue failure also initiated on the tension side of the specimen as identified from the compression shear lip. However, most failures initiated within the hybrid layer or in the region of the hybrid layer and dentin. Thereafter, the progression of failure occurred within the resin adhesive as evident from the portion of surface occupied by adhesive (A) in Figure 6(b). Nearest the biofilm exposure, the fracture surfaces of dentin were essentially free of adhesive resin as evident by open tubules. A magnified view of the fracture surface nearest the tensile surface for a biofilm treated specimen is shown in Figure 6(c). There is a transition in surface characteristics approximately 30 to 50 μm below the tensile surface (and biofilm treated) as outlined by the arrows. At much higher magnification, collagen fibrils are evident on the boundary of the lumens, suggesting localized demineralization. Beneath this transition line, the fracture surface had a greater degree of intact resin remaining on the surface.

Micrographs of the remaining unbroken interfaces after fatigue failure from water-aged and biofilm treated specimens are shown in Figure 6(d) and 6(e), respectively. Both of these micrographs were obtained from the tensile surfaces. In the water-aged specimens (Figure 6(d)) microcracking was often identified in the resin composite, adjacent to the interface. The microcracks were more commonly identified in the specimens subjected to larger cyclic stresses, and appeared at the boundary or within reinforcing particles (note arrows in Figure 6(d)). Specimens subjected to lower cyclic stresses, which correspondingly endured longer lives ($N_f = 1 \times 10^5$ cycles), also exhibited microcracking at the edge of the resin composite and localized within the resin adhesive, as well. There was no difference between characteristics of the control specimens that underwent one and 14 days of water aging. In the specimens treated with biofilm, there was some microcracking evident within the resin composite as well, about the boundary of individual particulate reinforcements as evident in Figure 6(e). The most apparent characteristic of these specimens was demineralization of the tubule lumens as noted by the increase in lumen diameter and reduction of the peritubular

cuff thickness. There were also faint cracks evident within the hybrid layer, or at the intersection of dentin and the resin adhesive, which are evident and highlighted by the arrows in Figure 6(e).

NanoDMA scanning was conducted of the unbroken bonded interfaces after fatigue failure of the specimens. Scanning was conducted on the side of the specimen just beneath the surface of maximum principal stress, at the neutral axis, and along the compressive side. Complex modulus distributions of the intact interface for water-aged (one day) and biofilm exposed specimens are shown in Figures 7(a) and 7(b), respectively. Both of these maps represent a $50 \times 50 \mu\text{m}^2$ scanned window of evaluation with the adhesive interface extending horizontally. Clearly evident in both of these maps are the dentin (D), resin adhesive (A) and the resin composite (C). The reinforcing particulates of the resin composite are very distinct in the composite. In addition, the interfaces between the dentin, resin adhesive and resin composite are evident by the sharp reduction in modulus to between 3 and 5 GPa. The complex modulus for dentin in the water-aged specimens ranges primarily between 10 and 20 GPa, except along the peritubular cuffs where the modulus ranges between roughly 25 and 35 GPa. In the biofilm treated specimens there was evidence of degradation of dentin, which was identified by a reduction in the complex modulus of dentin most immediate to the surface of biofilm exposure. In the map of Figure 7(b) biofilm exposure occurred along the right side as annotated by the arrows. The elastic modulus of the intertubular region adjacent to this surface has been reduced to between 5 and 10 GPa, but this zone is limited to approximately 10 to 20 μm below the exposed surface. There was no apparent reduction in the complex moduli of the composite in any of the specimens examined.

DISCUSSION

Adhesive bonds to dentin may undergo degradation *in vivo* by hydrolytic processes that attack the collagen matrix [e.g. Tjäderhane et al., 2012], via plasticization of the polymer [e.g. De Munck et al., 2009; Spencer et al., 2010] or by the acid production of biofilms [e.g. Sakaguchi, 2005; Ten Cate, 2006]. During this concert of processes the margins are subjected to cyclic stresses that result from mastication and temperature changes. Surprisingly though, past *in vitro* evaluations of the resin-dentin interface have been primarily conducted using quasi-static loading, and have not assessed the contributions of oral bacteria to the degradation in mechanical properties. To the authors' knowledge, this investigation is the first to study the fatigue strength of the resin-dentin bonded interface after biofilm exposure, and to quantify the degree of reduction in durability with respect to water aging.

Results of the experiments showed that exposure to biofilm caused a significant reduction in strength and resistance to fatigue with respect to an equivalent period of water aging. There was a 50% reduction in the interface strength under quasi-static loading and a 30% reduction in the apparent endurance limit. For comparison, the apparent endurance limits of homogenous specimens of coronal dentin and resin composite examined under similar conditions are 43 MPa [Arola and Reprogel, 2006] and 48 MPa [Mutluay et al., 2012], respectively. These values are over three times greater than that of the resin-dentin interface after water aging, and over 4 times greater than after exposure to biofilm (10 MPa). Clearly the resin dentin-bonded interface is the weakest link under cyclic loading [Spencer et al., 2010] and exposure to biofilm further increases its susceptibility to fatigue failure.

Quantifying the reduction in fatigue strength is a valuable form of assessment, but the reduction in life and reliability of the bonded interface are perhaps more relevant to clinical practice. The fatigue life distribution of the biofilm treated specimens (Figure 5(b))

exhibited a considerably larger degree of scatter than the controls as evident from the lower coefficient of determination for the power law model ($R^2=0.53$). That implies that exposure to *S. mutans* bacteria not only resulted in a substantial degradation of strength, but also caused a reduction in reliability. Moreover, if the cyclic stresses transmitted across the bonded interface *in vivo* are between 15 and 20 MPa (Arola et al., 2001; Lin et al., 2008), the results in Figure 5(b) show that the life of the interface exposed to biofilm is reduced to approximately 2% (i.e. $1/50^{\text{th}}$) of that achieved after water aging only. The 14-day continuous exposure to biofilm was admittedly aggressive. Nevertheless, the results clearly distinguish that resistance to degradation by biofilm is an important consideration in future studies aimed at addressing resin-dentin bond durability.

In comparison to the application of microtensile testing for evaluating resin-dentin bonds, experimental evaluations of the fatigue behavior are rather scant [Drummond et al., 1996; Frankeberger et al., 2003; Staninec et al., 2008, Belli et al., 2010]. Most of these studies adopted a fatigue limit of 1×10^5 cycles or less. With an estimated 500 to 750 cycles of mastication per year [Anusavice, 1996], the aforementioned definition corresponds to far less than 1 year of oral function. If the goal of restorative dentistry is to support lifelong oral health, then a longer period of assessment may be more appropriate for evaluating durability. Staninec et al. [2008] characterized the fatigue strength of adhesive bonds to dentin in HBSS and four-point flexure up to one million cycles. That study also involved SE Bond, but the apparent endurance limit (25 MPa at 1×10^6 cycles) is nearly twice that obtained for the water aged control specimens evaluated presently. The lower strength obtained in the present investigation is expected to be due to differences in the bonding area. Staninec et al., [2008] used a single bonded interface with area (0.76 mm^2), which is less than one tenth that of the twin interface specimens (i.e. 8 mm^2). There are many advantages to a larger specimen size, but the bond strength strengths are generally lower due to the greater population of defects [Phrukkanon et al., 1998; Burrow et al., 2004]. The prior study also used Filtek Z-250, which has smaller average filler size and could be an important contributor as well due to the initiation of interface failure at the boundary of larger particulates (Figure 6(d)).

It is important to address the mechanisms contributing to the differences in strength and durability between the control and biofilm treated specimens. Although exposure to bacteria was expected to cause a reduction in fatigue strength, the greater question pertains to the cause(s). Was failure a result of the comparatively weak adhesive? Evaluation of the fracture surfaces showed that failure of all the control specimens (100%) initiated on the tension side and within the resin composite, adjacent to the interface (Figure 6(d)). Cause of failure in the control specimens appeared to consist of debonding between the resin and reinforcing particles, and fracture of larger particulates. Cyclic loading simply enabled coalescence and growth of these defects, until they facilitated fracture. Failure in the biofilm treated specimens also occurred in tension, but initiated predominately within hybrid layer and adjacent dentin (Figure 6(e)). Only three of the 20 specimens had characteristics suggesting that failure initiated within the composite.

The surface morphology and exposed dentin tubules showed that acid production of the biofilm caused extensive demineralization at the surface (Fig. 6(e)), just beneath the incubated biofilm (Figure 2(b)). Nevertheless, measures of the elastic modulus distribution obtained from nanoDMA revealed that the depth of the “acid-affected” zone was less than $50 \text{ }\mu\text{m}$ (Figure 7(b)). Those observations suggest that the reduction in strength and durability of the biofilm treated specimens resulted primarily from focused demineralization of dentin about the interface, and a reduction in the integrity of the adhesion between the resin adhesive and dentin. The degradation was sufficient to enable debonding under cyclic loading at lower cyclic stress. Yet, previous investigations on the exposure of resin

composites to biofilms reported that *S. mutans* caused a significant increase in their surface roughness [Beyth et al., 2008; Fúcio et al., 2008]. One month of exposure caused an increase in roughness on the order of a few tens of nanometers, and only minimal changes in surface hardness. Topographical changes in the resin composite surfaces were not assessed in the present investigation, but based on the microscopic examinations it was apparent that the greatest changes occurred to the dentin. In addition to degradation of the collagen and mineralized dentin, the poorly polymerized portions of the resin adhesive are susceptible to degradation in both the water and acidic environments [Bail 2012, Vaidyanathan 2009]. The hybrid layer often contains areas with lower degree of conversion and poor cross-linking density as a result of residual unbound water and solvents [Cadenaro et.al 2008]. Thus, a portion of the observed changes in fatigue resistance after 14 day exposure to water and biofilm may be attributed to the degradation of the polymer portion of the hybrid layer. Changes to the polymer were not characterized in the present investigation. Therefore, it appears necessary to explore the chemical changes in the resin composite, adhesive and dentin in future studies concerning degradation in interface durability with biofilm exposure.

The twin bonded interface specimen adopted for this investigation is novel and may appear complicated in comparison to the conventional microtensile specimen with single interface. But the specimen is actually quite simple due to its symmetry, as the twin interfaces experience exactly the same nominal normal stress distribution. Furthermore, the tensile normal stress distribution (at the surface) resulting from 4-point flexure is essentially identical to that that would result from uniaxial loading as shown in Figure 4(b). There are other factors worth discussing. For example, the stress state across the interface is not uniform (Figure 3(c)), and the largest stress exists within the dentin, adjacent to the interface. But that is a criticism of the micro- and macro-tensile tests as well, and even the bonded interface *in vivo*, which stems from the compliance of the resin adhesive (Figure 3(b)). Another point of relevance is the proximity of the interior loading pins to the bonded interfaces. The chosen specimen and loading configuration reduces the volume of resin necessary for preparing these specimens. The interior loading pins contact the specimens at a distance in which, according to Saint-Venant's Principle [Popov, 1978], contact stresses should not be ignored. Results from the finite element analysis showed that the influence of contact stresses is negligible on the tensile side of the interface (Figure 3(c)), i.e. where failure initiated, and the largest deviation in the flexure stress from uniformity is caused by the interface (Figure 4(a)). Taken together, though there may be some concerns regarding the new specimen design, there are many attractive features.

Four-point flexure was chosen over shear or uniaxial tensile loading for evaluating the mechanical behavior of the interface. One might question whether this mode of loading has any clinical relevance. Indeed, flexure was chosen specifically for the stress state. Reported finite element studies show that the stress distribution resulting from occlusal loading of teeth with bonded composite restorations have maximum values at the surface and line angles and gradients extending beyond that [Arola et al., 2001; Asmussen and Peutzfeldt, 2008]. There are also a number of benefits to its application, namely it provides a region of uniform normal stress between the two inner contacts, and it promotes a stress gradient with maximum value at the surface. This stress gradient may amplify the affects of degradation caused by biofilm at the surface, but it is a clinically relevant amplification for the aforementioned reasoning. One might fear that the specimen geometry and loading profile does not follow a standard, and the aspect ratio of the beam does not conform to that of standardized approaches. There is presently no standard for evaluating the durability of the resin-dentin interface. And as evident in Figure 4(a), the stress distribution that develops across the interface is non-uniform, and would change with alternate combination of resin adhesive and composite [Misra et al., 2004]. Another concern relates to the degradation of dentin by the biofilm and how that might change the stress state in dentin or at the interface.

While there were rather distinct signs of demineralization at the surface of dentin (Figure 6(d)), results from nanoDMA showed that the demineralized region did not extend more than 25 to 35 μm beneath. Undoubtedly there were changes in the micro-mechanical aspects of load transfer along the interface of the biofilm treated specimens with fatigue [Singh et al., 2011], but these are beyond the scope of the present investigation.

Apart from concerns related to the experimental approach, there are limitations to the investigation that should be considered in future studies. The biofilm was incubated on the molded specimens in a quiescent environment. It would be more clinically relevant for the specimens to be subjected to cyclic loading during the incubation process. One contributing factor for the limited depth of the “acid-affected” zone is that the biofilm was incubated without stress. Cyclic stresses would facilitate pumping of acid produced by biofilm within the lumens [Ivancik et al., 2011] and along the boundary of dentin and the compliant adhesive [Wood et al., 2008]. Furthermore, the biofilm attack model followed that of previous studies [e.g. Hara et al., 2006; Aires et al., 2008; Cenci et al., 2009] and lasted for 14 days. It may be more clinically relevant to consider intermittent intervals of biofilm exposure and cyclic loading, with potential modifications to the period of bacterial exposure. Furthermore, the bonded interface specimens were freshly prepared, intact and without noticeable microgaps. Margins *in vivo* often develop microgaps with function, which could harbor bacteria and facilitate acid attacks to deeper dentin. Further studies are needed to investigate these aspects of dentin bonding in combination with cyclic loading. Based on results of the experiments with biofilm, remineralization appears to be an essential component of the strategies for preventing bond degradation [Liu et al., 2011]. The twin bonded interface approach may serve as a useful platform to study proposed solutions for the maintenance and repair of adhesive bond integrity.

CONCLUSION

In summary, a 14 day exposure to *S. mutan* biofilm resulted in a significant reduction in both quasi-static flexure strength and fatigue strength of the resin-dentin interface in comparison to those properties after an equivalent period of water aging. There was no apparent degradation in the properties of the resin composite within the 14 days of water aging or exposure to biofilm. Thus, the reduction in bonded interface durability was caused by degradation of dentin and adjacent hybrid layer, most notably by loss of mineral caused by the acid production of biofilm. Although the methods used in this *in vitro* study for studying the bonded interface durability are not complicated, this investigation identified that efforts to extend the longevity of resin composite restorations will require strategies to maintain the integrity of dentin.

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a)

b)

c)

Figure 1.

Preparation of the resin-dentin bonded interface specimens. a) obtaining a section of coronal dentin for placement in the mold (b) development of the bonded interface. Denoted are the resin composite (C) and dentin (D). Primer and adhesive are applied to the dentin beam outside of the molding fixture, and light-cured after the beam is placed in the fixture. The resin composite is applied incrementally on both sides of the dentin and light-cured. Slicing of the molded sections is then performed according to the dashed lines to obtain two interface specimens with final dimensions of approximately $2 \times 2 \times 12 \text{ mm}^3$. Selected molded sections are exposed to biofilm prior to slicing, c) The four-point flexure configuration for evaluating the bonded interface in quasi-static and cyclic loading. Denoted are the resin

composite (C), dentin (D) and the twin bonded interfaces comprised of resin adhesive and hybrid layer (A+H). Note that the surface exposed to biofilm of the treated specimens is subjected to tension.

Figure 2. Incubation of biofilm on the bonded interface specimens. a) SEM micrograph of the biofilm after 14 day incubation, b) Live/dead staining of the biofilm on the specimen from (a). Live bacteria were stained green, and dead bacteria were stained red. Live and dead bacteria in proximity to each other yielded yellow/orange staining.

Figure 3.

The Finite Element Analysis (FEA) for flexure and tensile loading of the bonded interface specimens. Consistent with Figure 1, the resin composite (C), dentin (D) and the twin bonded interfaces comprised of resin adhesive and hybrid layer (A+H) are highlighted. a) Schematic of simulated flexure loading and outline of region of interest from the axis of symmetry to beyond the interior loading pin. The full beam was modeled and results from the region of interest are shown in (b) and (c). The specimen was subjected to a 10 Newton flexure load. b) Normal strain distribution in the x direction resulting from flexure loading. Red = tensile strain and Blue = compressive strain. c) Normal stress distribution in the x direction resulting from flexural loading.

Figure 4.

Evaluation of the stress distribution across the bonded interface.

a) Comparison of the normal stress distribution in flexure from the finite element model and that estimated using beam theory. The stresses are plotted from the specimen's center ($x=0$) along the tensile surface (i.e. the region of maximum normal stress) to the position of pin contact ($x = 1.5$ mm). b) Comparison of the normal stress distribution resulting from simulated flexural loading (in (d)) with that resulting from simulated tensile loading.

a)

Condition	A (MPa)	B	R ²
1 d	55.4	-0.090	0.92
14 d	42.7	-0.073	0.80
14 d w/B	33.3	-0.075	0.53

b)

Figure 5. Strength of the bonded interface specimens after water aging and biofilm exposure. a) flexure strength resulting obtained from quasi-static loading to failure. Columns with different letters are significantly different (p 0.000). b) Stress life fatigue diagram from cyclic loading to failure. Data points with arrows represent specimens that did not fail. The power law constants (A, B) describing the fatigue life distributions and coefficient of determination (R²) are also provided.

Figure 6.

Microscopic evaluation of the bonded interface specimens after fatigue failure. a) fracture surface of water aged specimen (1 day). The tension surface is upright and a compression shear lip (S) is evident near the bottom. Common for most water-aged specimens, the majority of the fracture surface is occupied by composite. b) fracture surface of specimen exposed to biofilm with tension surface upright. Failure of these specimens initiated in the hybrid layer or adjacent dentin and then continues into the resin adhesive; H=hybrid layer, A=resin adhesive. c) Magnified view of the tension surface from a biofilm exposed specimen. Note the discoloration in the dentin closest to the biofilm-exposed surface (top) as indicated by the arrows. The fracture surface nearest biofilm exposure exhibited evidence of demineralization including larger lumens and exposed collagen. d) The remaining intact interface (tensile side) from a specimen aged in water for 24 hours and subjected to cyclic loading to failure; D=dentin, A=resin adhesive and C=resin composite. Microcracks are evident in the resin composite at the boundary of large filler particles (arrows). e) The remaining intact interface (tensile side) from a specimen exposed to biofilm. Note the degradation of dentin including demineralized lumens and small cracks at the adhesive-dentin interface (arrows).

Figure 7.

Complex modulus (E^*) distributions determined from nanoDMA evaluation of selected specimens after cyclic loading. Each scanned area corresponds to a $50\ \mu\text{m} \times 50\ \mu\text{m}$ nanoDMA analysis window and the adhesive interface is oriented horizontally. The windows represent portions of the interface subjected to cyclic tension. D= dentin, A=Adhesive, C= resin composite, p= peritubular cuff, f= filler particles of the AP-X resin composite.

a) interface of specimen after 24 hour water aging, b) interface after 14 day exposure to biofilm. Note that the surface exposed to biofilm is to the right (arrows, B) and shows a region of lower relative modulus.