Published in final edited form as:

Dent Mater. 2014 August ; 30(8): 926–935. doi:10.1016/j.dental.2014.05.009.

Hydrophilicity of dentin bonding systems influences *in vitro* *Streptococcus mutans* biofilm formation

Eugenio Brambilla¹, Andrei Ionescu¹, Annalisa Mazzoni², Milena Cadenaro², Massimo Gagliani¹, Monica Ferraroni³, Franklin Tay⁴, David Pashley⁴, and Lorenzo Breschi⁵

¹Department of Health Sciences, University of Milan, Milan, Italy

²Department of Medical Sciences, University of Trieste, Trieste, Italy

³Dipartimento di Medicina del Lavoro, University of Milan, Milan, Italy

⁴Department of Oral Biology, College of Dental Medicine, Georgia Health Sciences University, Augusta, Georgia, USA

⁵Department of Biomedical and Neuromotor Sciences, DIBINEM, University of Bologna - Alma Mater Studiorum, Bologna, Italy and IGM-CNR, Unit of Bologna c/o IOR, Bologna, Italy

Abstract

Objectives—To evaluate *in vitro* *Streptococcus mutans* (*S. mutans*) biofilm formation on the surface of five light-curing experimental dental bonding systems (DBS) with increasing hydrophilicity. The null hypothesis tested was that resin chemical composition and hydrophilicity does not affect *S. mutans* biofilm formation.

Methods—Five light-curing versions of experimental resin blends with increasing hydrophilicity were investigated (R₁, R₂, R₃, R₄ and R₅). R₁ and R₂ contained ethoxylated BisGMA/TEGDMA or BisGMA/TEGDMA, respectively, and were very hydrophobic, were representative of pit-and-fissure bonding agents. R₃ was representative of a typical two-step etch- and-rinse adhesive, while R₄ and R₅ were very hydrophilic resins analogous to self-etching adhesives. Twenty-eight disks were prepared for each resin blend. After a 24 h-incubation at 37 °C, a multilayer monospecific biofilm of *S. mutans* was obtained on the surface of each disk. The adherent biomass was determined using the MTT assay and evaluated morphologically with confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM).

Results—R₂ and R₃ surfaces showed the highest biofilm formation while R₁ and R₄ showed a similar intermediate biofilm formation. R₅ was more hydrophilic and acidic and was significantly less colonized than all the other resins. A significant quadratic relationship between biofilm

© 2004 Academy of Dental Materials. Published by Elsevier Ltd. All rights reserved.

CORRESPONDING AUTHOR: Prof. Lorenzo Breschi DDS, PhD, FADM, Dental School - Department of Biomedical and Neuromotor Sciences, DIBINEM, University of Bologna - Alma Mater Studiorum, Via San Vitale, 59, 40125, Bologna, Italy. Tel: +39-051-20 88139; Fax: +39-051-22 5208; lorenzo.breschi@unibo.it.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

formation and hydrophilicity of the resin blends was found. CLSM and SEM evaluation confirmed MTT assay results.

Conclusions—The null hypothesis was rejected since *S. mutans* biofilm formation was influenced by hydrophilicity, surface acidity and chemical composition of the experimental resins. Further studies using a bioreactor are needed to confirm the results and clarify the role of the single factors.

Keywords

Dentin Bonding Systems; Hydrophilicity; Biofilms; *Streptococcus mutans*

1. INTRODUCTION

Resin-based composites are increasingly used because of their excellent esthetic properties and improved mechanical characteristics [1, 2]. The evolution of polymer matrices and filler particles composition enhanced the performances of these materials [3–5] along with more reliable bonds to dental hard tissues. Nevertheless, the main reason for failure of resin composite restorations is still secondary caries occurring at the interface between the composite material/dentin bonding systems (DBS) and enamel and dentin [6–11].

As the development of such lesions is mainly related to the effect of acids and enzymes produced by bacteria colonizing the interface, the interactions between the surface of resin composites, DBS and the overlying biofilm greatly influence the lifespan of the adhesive restorations. A relevant problem is that resin composite and DBS surfaces are more heavily colonized by oral biofilms than surfaces of other restorative materials (such as amalgam, glass-ionomer cements and ceramics), as well as sound enamel surfaces [12–14].

Dentin bonding systems are blends of hydrophilic and hydrophobic monomers with the ability to couple the hydrophobic resin composite materials to hydrophilic surfaces such as dentin or enamel. They may be classified as etch-and-rinse or self-etching adhesives. These adhesives may be further classified as multi-step (i.e. three-step etch-and-rinse and two-step self-etching) or simplified by combining the number of steps required for the clinical application (i.e. two-step etch-and-rinse and one-step self-etching) [15–17]. Since simplified formulations involve mixing of nonsolvated adhesives with solvated primers (i.e. two-step etch-and-rinse) or with self-etching primers (i.e. one-step self-etch), DBS simplification strongly increases the hydrophilicity of the mixture and of the bond [18]. It has been previously shown that the chemical composition of DBS influences bacterial colonization. The results suggest that variations in the chemical structure of the monomers, solvents or application techniques can extensively influence biofilm formation even when a DBS does not contain any specific antibacterial formulation [19–25].

Previous studies investigated the effect of commercially available DBS on cariogenic bacteria colonization. Pinheiro *et al.* [26] studied *S. mutans* biofilm formation on the surface of different DBS. The authors concluded that different materials produce diverse colonization levels in relation to their variable chemical composition, solvent and application technique. Comparison between etch-and-rinse and self-etching adhesives

showed lower *S. mutans* colonization for the latter, when compared to the etch-and-rinse approach [27],[28]. Additionally, discrepancies in bacterial growth induced by different one-step self-etching adhesives and self-etching primers have been reported [29].

Experimental resins that cover a wide range of hydrophilicity and other properties of contemporary DBS have been formulated (R₁-R₅); these resins rank from very hydrophobic to very hydrophilic, as manifested by their solubility parameters [17]. Previous studies have evaluated their tensile strength, modulus of elasticity, degree of conversion, influence of solvent content, water sorption and solubility [17, 30–36]. However, no data is available regarding the influence of the hydrophilicity of these experimental resin blends on surface biofilm formation. Since *S. mutans* is considered to be one of the most important microorganisms responsible for primary and secondary caries [37–40], the aim of the present study was to investigate the influence of experimental DBS chemical composition and hydrophilicity on *S. mutans* colonization *in vitro*. The null hypothesis tested was that hydrophilicity of DBS does not affect *S. mutans* biofilm formation.

2. MATERIALS AND METHODS

2.1 Specimen preparation

All reagents and multi-well plates used in the present study were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Five light-curing versions of neat experimental resin blends with increasing hydrophilicity were investigated (R₁, R₂, R₃, R₄ and R₅). Their compositions are listed in Table 1. All blends included 0.25% camphorquinone and 1% 2-ethyl-dimethyl-4-aminobenzoate as the photoinitiator and accelerator, respectively. Resin blend R₁ and R₂ are similar to nonsolvated hydrophobic resins used in the formulation of the bonding agent of three-step etch-and-rinse and two-step self-etching adhesives. Resin blend R₃ represents a typical two-step etch-and-rinse adhesive. Resin blends R₄ and R₅ contain methacrylate derivatives of carboxylic and phosphoric acids, respectively, and they have the highest hydrophilicity, similarly to one-step self-etching adhesives. All resin blends were formulated in an increasing order of hydrophilicity based on their Hoy's solubility parameters (Table 1).

Twenty-eight disks of each experimental resin blend (6.4 ± 0.1 mm diameter; 1.00 ± 0.02 mm thick) were prepared by placing 45 μ L of resin on the bottom of modified 96-well polystyrene plates. The plates were sealed with impression material (Express™ 2 Light Body Standard, 3M ESPE, Seefeld, Germany), leaving a 2 mm void space between the lid and the wells. Then, two holes (3 mm diameter) were prepared on top of lid. The holes were sealed by plastic joints and connected by tubing to a nitrogen source. A constant flow of nitrogen was maintained inside the plates for 20 min to create an oxygen-free environment. The resin blend in each well was cured through the bottom of the plate for 80 sec, using a quartz-tungsten halogen light-curing unit (Spectrum 800, Dentsply International Inc., York, PA, USA). The light-curing unit was set at a power level of 800 mW/cm². This procedure was used to obtain a smooth resin surface and a perfect fit between the resin disk and the well, overcoming the formation of an oxygen-inhibited layer and the need for surface finishing.

Each plate was stored under light-proof conditions for 24 h at 37 °C to allow complete polymerization of the resins. Then 200 µL of sterile phosphate-buffered saline (PBS) were added to each well. Plates were stored at room temperature for an additional 7 days, allowing unreacted monomers to leach out. To remove those compounds, each well was rinsed twice a day with 200 µL of sterile PBS. The plates were sterilized with a chemical peroxide-ion plasma sterilizer (STERRAD, ASP, Irvine, CA, USA) for 60 min at a maximum temperature of 45 °C to prevent heat-induced modification of the resin surface.

2.2 Bacteria

All the culture media were obtained from Becton-Dickinson (BD Diagnostics-Difco, Franklin Lakes, NJ, U.S.A). *Streptococcus mutans* ATCC 25175 was cultured on Mitis Salivarius Bacitracin agar. The plates were incubated at 37 °C for 48 h in a 5% supplemented CO₂ environment and a pure suspension of the microorganism in Brain Heart Infusion (BHI) was obtained from these plates after an incubation time of 12 h at 37 °C in a 5% supplemented CO₂ environment. *Streptococcus mutans* cells were harvested by centrifugation (2,200 rpm for 5 min at 19 °C), rinsed twice with PBS and resuspended in the same buffer. The suspension was then sonicated to disperse bacterial chains (Sonifier model B-15, Branson, Danbury, CT, USA, operating at 40 W energy output). The optical density was adjusted to 0.3 OD units at 550 nm using a spectrophotometer (Genesys 10-S, Thermo Spectronic, Rochester, NY, USA), which corresponds to a microbial concentration of 3.65×10^8 cells/mL.

2.3 MTT assay reagents

A (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stock solution was prepared by dissolving 5 mg/mL of MTT in sterile PBS. A N-methylphenazonium methyl sulphate (PMS) stock solution was prepared by dissolving 0.3 mg/mL of PMS in sterile PBS. The solutions were stored at 2 °C in light-proof conditions until the day of the experiment. A fresh measurement solution (FMS) was prepared by mixing 1 mL of MTT stock solution, 1 mL of PMS stock solution, and 8 mL of sterile PBS. A lysing solution (LS) was prepared by dissolving 10% V/V of sodium dodecyl sulphate and 50% V/V of dimethylformamide in distilled water.

2.4 Biofilm development and MTT assay

Sixteen disks prepared from each experimental resin blend were used for this test. Twenty microliter of the *S. mutans* cell suspension and 180 µL of sterile BHI supplemented with 5% sucrose were placed in each well of the plates. The mixture was incubated for 24 h at 37° C in a 5% supplemented CO₂ environment, as required for the development of a multilayer biofilm [41]. In brief, the wells were carefully washed three times with sterile PBS to remove non-adherent cells. One hundred microliter of FMS was then placed in each well and the plates were incubated for 3 h at 37 °C in light-proof conditions. During incubation, microbial redox systems converted the yellow MTT salt to insoluble purple formazan. The unreacted FMS was carefully removed and formazan crystals were dissolved by adding 100 µL of LS to each well and by incubating for 1 h at room temperature under light-proof conditions. Ninety microliter of suspension were obtained from each well and the optical

density at 550 nm was measured using a microplate reader (Genesys 10-S, Thermo Spectronic, Rochester, USA).

2.5 Laser confocal microscopy (CLSM) and scanning electron microscopy (SEM)

Six disks from each resin blend were analysed using CLSM and six disks examined using SEM. After the 24-h incubation time, the biofilm growing on the disks was gently washed three times with PBS to remove non-adherent cells and stained using the FilmTracer™ LIVE/DEAD® Biofilm Viability Kit for microscopy (Invitrogen Ltd., Paisley, United Kingdom). The fluorescence from live stained cells adherent to the test samples was observed using a CLSM (Leica TCS SP5, Leica Microsystems, Wetzlar, Germany). Ten randomly selected image stack sections were recorded for each biofilm specimen. Confocal images were obtained using a 40× (NA 1.25) oil immersion objective and digitalized by using the Leica Application Suite Advanced Fluorescence Software (LAS AF, Leica microsystems, Wetzlar, Germany), at a resolution of 1024×1024 pixels, with a zoom factor of 3.0. For each image stack, a maximum-intensity projection (MIP) reconstruction was obtained.

Using the same incubation time, the 6 disks selected for SEM observation were gently washed three times with PBS to remove non-adherent cells and placed in a 2% glutaraldehyde fixative solution for 1 week. The specimens were then transferred to 70, 80, 85, 90, and 95% (v/v) ethanol solutions for 24 h each, and finally in a 100% ethanol solution for 48 h. Finally, the specimens were critical point dried (Critical-point Dryer, EMS 850, Hatfield, PA, USA), mounted on stubs with conductive glue, sputter-coated (JEOL FFC-1100, Japan), and observed with a scanning electron microscope (JEOL JSM -5300, Japan) at a magnification of 2000×.

2.6 pH analysis

Resin disks were obtained at the bottom of 96 well plates by placing 45 µL of resin on the bottom of modified 96-well polystyrene plates and by polymerizing in a nitrogen atmosphere as specified in paragraph 2.1. Then, resin specimens underwent rinsing (as previously described in paragraph 2.1) and pH measurements were conducted on 4 specimens for each resin blend using a ROSS micro-pH electrode (Thermo Scientific, Waltham, MA, USA) which fitted inside the wells of 96-well plates. Two-hundred microliters of distilled water or PBS or BHI solution were inserted inside the specimen-containing wells and measurements were performed immediately (time=0h) or after 3h, 6h, 12h and 24h. After that, solutions were discarded and plates were rinsed three times with distilled water and 200 µl of new solutions were inserted again inside the wells; measurements were performed immediately (time=24h, after rinsing). Every 20 measurements the electrode was checked against standard solutions and, if necessary, recalibrated.

2.7 Statistical analysis

Statistical analysis was performed with Stata v.10 software (Stata v.10, StataCorp., College Station, TX, USA). Normal distribution of data was checked using the Shapiro-Wilk test and homogeneity of variances was verified using Bartlett's test. One-way ANOVA ($p < 0.05$) was

performed and Student-Neumann-Keuls post-hoc test was used to assess differences among the resin groups. The level of significance (α) was set to $p < 0.05$. The existence of a relationship between Hoy's parameters and biofilm formation on the surfaces of the different resin blends was evaluated by fitting the data series with a quadratic function.

3. RESULTS

The means and standard errors of the MTT assay on the 5 experimental resin blends are reported in Figure 1. Resin blends R_2 and R_3 showed the highest *S. mutans* colonization. Resin blends R_1 and R_4 showed similar intermediate biofilm formation while R_5 surfaces were significantly less colonized than the other resin blends, although its composition differed from R_3 only for the presence of an acidic phosphate monomer Bis[2-(methacryloyloxy)ethyl]phosphate ($p < 0.001$). Similarly, R_4 showed a significantly lower biofilm formation than R_2 , which contained the same monomers except for the acidic carboxylate monomer di(hydroxyethylmethacrylate) ester of 5-(2,5-dioxotetrahydrofurfuryl)-3-methyl-3-cyclohexane-1, 2'-dicarboxylic acid ($p < 0.001$).

A quadratic function (Fig. 2) provides a good correlation model for the relationship between Hoy's solubility parameters for total cohesive forces and biofilm formation on the surfaces of the different resin blends ($p < 0.025$).

Figure 3 shows live/dead stain images of 24-h incubation biofilms. Biofilms developed on the surfaces of R_2 and R_3 resin blends showed almost complete coverage with mostly live microorganisms. Specimens derived from resin blend R_5 exhibited very low biofilm formation with a prevalence of red-stained (i.e. dead) microorganisms. Biofilms grown on the surfaces of R_1 and R_4 resin blends showed intermediate features.

Scanning electron microscopy images of biofilms grown on the surface of different resin blends are shown in Figure 4. The SEM results confirm the CLSM morphologic observations.

Table 2 shows the results of the pH analysis. R_5 was significantly more acidic than all other resin blends irrespective with the solution tested and at any tested time ($p < 0.0001$). The first measurement time indicated that pH values of R_5 were lowest when assessed in distilled water; they were partially buffered by PBS and almost completely buffered by BHI, while R_4 was less acidic and was completely buffered by BHI. Nevertheless, R_5 was able to overwhelm the buffering capacity of both PBS (after 6h) and BHI (after 12h). The measurement performed after inserting new solutions inside the wells indicated that the new PBS and BHI solutions were again able to buffer almost completely the pH values of the wells containing R_5 disks, while R_5 showed lowest pH values in the new distilled water solution.

4. DISCUSSION

Reduction of biofilm formation on the surface of resin-based materials is considered an important target to reduce secondary caries risk and improve the longevity of restorations. Previous studies investigating biofilm formation [12] were performed using the agar

diffusion test. In contrast, the method employed in the present study directly evaluated adherent biomass in experimental conditions that simulated *in vivo* clinical conditions. The results of the present study warrant rejection of the null hypothesis that hydrophilicity of DBS does not affect *S. mutans* biofilm formation. Indeed, differences in chemical composition and hydrophilicity of the experimental DBS significantly influenced *in vitro* *S. mutans* biofilm formation. Interestingly, reduced *S. mutans* biofilm formation was observed as the hydrophilicity of the experimental resin blends increased, with a relationship described by a quadratic function.

Indeed, the chemical composition of DBS and in particular the presence of acidic polymers may have had an important role in determining *S. mutans* biofilm formation. As confirmed by the pH measurements (Table 2), lower biofilm formation (R_4 and R_5) was also related to the more acidic resins which lead to the lower pH of the broth solution by the resin polymers. Resin blend R_5 , being the most hydrophilic and the most acidic (pH = 3.2) resin examined, showed the lowest bacterial colonization. Resin blend R_5 showed a significant reduction in *S. mutans* biofilm formation when compared to R_3 , which was characterized by the same composition, except for containing only 40% BisGMA instead of 70%, and containing 30% bis[2-(methacryloyloxy)ethyl]phosphate (BisMP), an acidic phosphate monomer. Similarly, R_4 (an acidic resin which contained 40 wt% BisGMA, 28.75% TEGDMA and 30 wt% for the acidic carboxylate monomer, dihydroxy ester of 3a, 4, 5, 7a-tetrahydro-7-methyl-5-(tetrahydro-2,5-dioxo-3-furanyl)-1,3-isobenzofurandione) produced significantly lower biofilm formation than did R_2 (a neutral resin).

Streptococcus mutans colonization is considered an important step in dental plaque formation as its presence increases the pathogenicity of the biofilm [42–44]. Biofilm formation is also influenced by the degree of conversion (DC) and surface roughness (SR) of resinous materials [45–49]. Low DC of DBS results in reduction of the mechanical properties of the adhesives [50]. Simplified DBS exhibited lower DC than conventional adhesives (three-step etch-and-rinse and two-step self-etching systems) [51, 52]. *In vitro* studies demonstrated that lower DC resulted in higher *S. mutans* biofilm formation [53]. Since hydrophilicity is correlated with DC, we speculate that DC may be an important parameter in determining the different extents of biofilm formation shown in the present study. Previous studies by several of our coauthors, reported that the amount of water sorption by R_{1-5} increased with the δ_p value of resin [31], with the most hydrophilic R_5 , exhibiting the highest water absorption over time, reaching 16% in 24 h [31]. R_5 also exhibited the highest solubility of the five resins even though it showed the highest degree of conversion ($71.6 \pm 2.5\%$) of the five resins [31]. We speculate that R_5 leached acidic bis[2-(methacryloyloxy)ethyl]phosphate. This may be a possible explanation for the finding that this resin was able to overwhelm the buffering capacity of both PBS and BHI solutions over shorter time periods than the biofilm formation time tested in this study (24 h). Another possibility is that esterases from *S. mutans* hydrolyzed some of that monomer to phosphoric acid [54]. If the microenvironment above R_5 surfaces had a pH less than 4, *S. mutans* could not grow on R_5 because it was too acidic. Most self-etching adhesives containing phosphate derivatives of methacrylates produce acidic polymers (e.g. Xeno IV, Adper Easy Bond). One would expect that they too might inhibit bacterial growth.

Surface roughness (SR) has also been identified as a key parameter influencing bacterial adhesion and biofilm formation on oral surfaces until a threshold of approximately 0.2 μm [47, 59, 60]. The procedure used in the present study allowed us to obtain highly standardized surfaces (SR below 0.2 μm), regardless of the DBS formulation. The specimen preparation included an extended light-curing time applied to both surfaces of the disks to ensure the highest possible DC for each experimental resin formulation. The specimens were also cured in an oxygen-depleted atmosphere to prevent the formation of an oxygen-inhibited layer and avoid the need of a finishing procedure. Furthermore, the specimens underwent a 7-day washout period, which was designed to allow most of the unpolymerized resin monomers to leach out. This procedure partially avoided the contribution of unpolymerized monomers on bacterial growth [57, 61].

Whereas material properties can influence bacterial colonization, microbial metabolic activity may likewise modify the properties of the material surface [45, 62]. Organic acids produced by oral biofilms can soften resin-based materials by permeation and extraction of compounds from the polymer. These acids cause DBS modifications, such as increase in surface roughness. This renders the roughened resin surface more susceptible to biofilm formation [45, 62].

Although the salivary pellicle has a definitive impact on bacterial colonization, it is the underlying biomaterial that ultimately determines the extensiveness of biofilm formation [43, 63, 64]. In the present study, a salivary pellicle was not used so as to more distinctively identify the effects of resin surface chemical characteristics on biofilm formation. The use of a static reactor enabled us to obtain a monospecies biofilm after 24 h of incubation. This technique reduces the variability between and within biofilms [14].

In conclusion, the present study showed that lower *S. mutans* biofilm formation is related to the hydrophilicity and acidity of the adhesive blends. Further studies using a continuous flow bioreactor and multi-species biofilms are needed to confirm these findings.

Acknowledgments

The authors report no conflict of interest and wish to thank Mr. Aurelio Valmori for photographic assistance. The study was funded by grants from MIUR (Italy): FIRB RBAP1095CR to Breschi L (P.I.), PRIN 2009SAN9K5 to Breschi L (P.I.) and R21 DE 091213 (P.I. FRT).

References

1. Ferracane JL. Resin composite – state of the art. *Dent Mater.* 2012; 19:449–57.
2. Drummond JL. Degradation, fatigue, and failure of resin dental composite materials. *J Dent Res.* 2008; 87:710–9. [PubMed: 18650540]
3. Carvalho RM, Manso AP, Geraldeli S, Tay FR, Pashley DH. Durability of bonds and clinical success of adhesive restorations. *Dent Mater.* 2012; 28:72–86. [PubMed: 22192252]
4. Pashley DH, Tay FR, Imazato S. How to increase the durability of resin-dentin bonds. *Compend Contin Educ Dent.* 2011; 32:60–4. [PubMed: 21910364]
5. Stansbury JW. Dimethacrylate network formation and polymer property evolution as determined by the selection of monomers and curing conditions. *Dent Mater.* 2012; 28:13–22. [PubMed: 22192248]

6. Deligeorgi V, Mjor IA, Wilson NH. An overview of reasons for the placement and replacement of restorations. *Prim Dent Care*. 2001; 8:5–11. [PubMed: 11405031]
7. Fontana M, Gonzalez-Cabezas C. Secondary caries and restoration replacement: an unresolved problem. *Compend Contin Educ Dent*. 2000; 21:15–8. 21–4. 26 passim; quiz 30. [PubMed: 11199687]
8. Soncini JA, Maserejian NN, Trachtenberg F, Tavares M, Hayes C. The longevity of amalgam versus compomer/composite restorations in posterior primary and permanent teeth: findings From the New England Children's Amalgam Trial. *J Am Dent Assoc*. 2007; 138:763–72. [PubMed: 17545265]
9. Hannig C, Kupilas FJ, Wolkewitz M, Attin T. Validity of decision criteria for replacement of fillings. *Schweiz Monatsschr Zahnmed*. 2009; 119:328–38. [PubMed: 19485073]
10. Brunthaler A, Konig F, Lucas T, Sperr W, Schedle A. Longevity of direct resin composite restorations in posterior teeth. *Clin Oral Investig*. 2003; 7:63–70.
11. Hondrum SO. The longevity of resin-based composite restorations in posterior teeth. *Gen Dent*. 2000; 48:398–404. [PubMed: 11199613]
12. Beyth N, Domb AJ, Weiss EI. An in vitro quantitative antibacterial analysis of amalgam and composite resins. *J Dent*. 2007; 35:201–6. [PubMed: 16996674]
13. Zhang K, Melo MA, Cheng L, Weir MD, Bai Y, Xu HH. Effect of quaternary ammonium and silver nanoparticle-containing adhesives on dentin bond strength and dental plaque microcosm biofilms. *Dent Mater*. 2012; 28:842–52. [PubMed: 22592165]
14. Rosentritt M, Hahnel S, Groger G, Muhlfriedel B, Burgers R, Handel G. Adhesion of *Streptococcus mutans* to various dental materials in a laminar flow chamber system. *J Biomed Mater Res B Appl Biomater*. 2008; 86:36–44. [PubMed: 18098186]
15. Van Meerbeek B, Yoshihara K, Yoshida Y, Mine A, De Munck J, Van Landuyt KL. State of the art of self-etch adhesives. *Dent Mater*. 2011; 27:17–28. [PubMed: 21109301]
16. Breschi L, Mazzoni A, Ruggeri A, Cadenaro M, Di Lenarda R, De Stefano Dorigo E. Dental adhesion review: aging and stability of the bonded interface. *Dent Mater*. 2008; 24:90–101. [PubMed: 17442386]
17. Cadenaro M, Breschi L, Antonioli F, Navarra CO, Mazzoni A, Tay FR, Di Lenarda R, Pashley DH. Degree of conversion of resin blends in relation to ethanol content and hydrophilicity. *Dent Mater*. 2008; 24:1194–200. [PubMed: 18342363]
18. Tay FR, Pashley DH. Have dentin adhesives become too hydrophilic? *J Can Dent Assoc*. 2003; 69:726–31. [PubMed: 14653938]
19. Rolland SL, McCabe JF, Robinson C, Walls AW. In vitro biofilm formation on the surface of resin-based dentine adhesives. *Eur J Oral Sci*. 2006; 114:243–9. [PubMed: 16776774]
20. Ozer F, Unlu N, Karakaya S, Ergani O, Hadimli HH. Antibacterial activities of MDPB and fluoride in dentin bonding agents. *Eur J Prosthodont Restor Dent*. 2005; 13:139–42. [PubMed: 16180641]
21. Atac AS, Cehreli ZC, Sener B. Antibacterial activity of fifth-generation dentin bonding systems. *J Endod*. 2001; 27(12):730–3. [PubMed: 11771577]
22. Imazato S. Bio-active restorative materials with antibacterial effects: new dimension of innovation in restorative dentistry. *Dent Mater J*. 2009; 28:11–9. [PubMed: 19280964]
23. Korkmaz Y, Ozalp M, Attar N. Comparison of the antibacterial activity of different self-etching primers and adhesives. *J Contemp Dent Pract*. 2008; 9:57–64. [PubMed: 18997917]
24. Paradella TC, Koga-Ito CY, Jorge AO. In vitro antibacterial activity of adhesive systems on *Streptococcus mutans*. *J Adhes Dent*. 2009; 11:95–9. [PubMed: 19492710]
25. Imazato S. Antibacterial properties of resin composites and dentin bonding systems. *Dent Mater*. 2003; 19:449–57. [PubMed: 12837391]
26. Pinheiro SL, Soares HH, Ribeiro MC. Microbial contamination and inhibitory effect against *Streptococcus mutans* from fifth-generation bonding systems. *J Appl Biomater Biomech*. 2010; 8:52–5. [PubMed: 20740422]
27. Espejo LC, Simionato MR, Barroso LP, Netto NG, Luz MA. Evaluation of three different adhesive systems using a bacterial method to develop secondary caries in vitro. *Am J Dent*. 2010; 23:93–7. [PubMed: 20608299]

28. Walter R, Duarte WR, Pereira PN, Heymann HO, Swift EJ Jr, Arnold RR. In vitro inhibition of bacterial growth using different dental adhesive systems. *Oper Dent.* 2007; 32:388–93. [PubMed: 17695612]
29. Esteves CM, Ota-Tsuzuki C, Reis AF, Rodrigues JA. Antibacterial activity of various self-etching adhesive systems against oral streptococci. *Oper Dent.* 2010; 35:448–53. [PubMed: 20672730]
30. Yiu CK, King NM, Pashley DH, Suh BI, Carvalho RM, Carrilho MR, Tay FR. Effect of resin hydrophilicity and water storage on resin strength. *Biomaterials.* 2004; 25:5789–96. [PubMed: 15147825]
31. Ito S, Hashimoto M, Wadgaonkar B, Svizero N, Carvalho RM, Yiu C, Rueggeberg FA, Foulger S, Saito T, Nishitani Y, Yoshiyama M, Tay FR, Pashley DH. Effects of resin hydrophilicity on water sorption and changes in modulus of elasticity. *Biomaterials.* 2005; 26:6449–59. [PubMed: 15949841]
32. Malacarne J, Carvalho RM, de Goes MF, Svizero N, Pashley DH, Tay FR, Yiu CK, Carrilho MR. Water sorption/solubility of dental adhesive resins. *Dent Mater.* 2006; 22:973–80. [PubMed: 16405987]
33. Pashley DH, Tay FR, Carvalho RM, Rueggeberg FA, Agee KA, Carrilho M, Donnelly A, Garcia-Godoy F. From dry bonding to water-wet bonding to ethanol-wet bonding. A review of the interactions between dentin matrix and solvated resins using a macromodel of the hybrid layer. *Am J Dent.* 2007; 20:7–20. [PubMed: 17380802]
34. Cadenaro M, Breschi L, Rueggeberg FA, Suchko M, Grodin E, Agee K, Di Lenarda R, Tay FR, Pashley DH. Effects of residual ethanol on the rate and degree of conversion of five experimental resins. *Dent Mater.* 2009; 25:621–8. [PubMed: 19111335]
35. Cadenaro M, Breschi L, Rueggeberg FA, Agee K, Di Lenarda R, Carrilho M, Tay FR, Pashley DH. Effect of adhesive hydrophilicity and curing time on the permeability of resins bonded to water vs. ethanol-saturated acid-etched dentin. *Dent Mater.* 2009; 25:39–47. [PubMed: 18571228]
36. Cadenaro M, Antonioli F, Codan B, Agee K, Tay FR, de Dorigo ES, Pashley DH, Breschi L. Influence of different initiators on the degree of conversion of experimental adhesive blends in relation to their hydrophilicity and solvent content. *Dent Mater.* 2010; 26:288–94. [PubMed: 20018363]
37. Garcia-Godoy F, Hicks MJ. Maintaining the integrity of the enamel surface: the role of dental biofilm, saliva and preventive agents in enamel demineralization and remineralization. *J Am Dent Assoc.* 2008; 139 (Suppl):25S–34S. [PubMed: 18460677]
38. Kutsch VK, Young DA. New directions in the etiology of dental caries disease. *J Calif Dent Assoc.* 2011; 39:716–21. [PubMed: 22132583]
39. Russell RR. Changing concepts in caries microbiology. *Am J Dent.* 2009; 22:304–10. [PubMed: 20225475]
40. Fucio SB, Carvalho FG, Sobrinho LC, Sinhoreti MA, Puppim-Rontani RM. The influence of 30-day-old *Streptococcus mutans* biofilm on the surface of esthetic restorative materials--an in vitro study. *J Dent.* 2008; 36:833–9. [PubMed: 18621456]
41. Brambilla E, Ionescu A, Fadini L, Mazzoni A, Imazato S, Pashley D, Breschi L, Gagliani M. Influence of MDPB-containing Primer on *Streptococcus Mutans* Biofilm Formation in Simulated Class I Restorations. *J Adhes Dent.* 2013; 15:431–8. [PubMed: 23534020]
42. Steinberg D, Eyal S. Early formation of *Streptococcus sobrinus* biofilm on various dental restorative materials. *J Dent.* 2002; 30:47–51. [PubMed: 11741735]
43. Busscher HJ, Rinastiti M, Siswomihardjo W, Van der Mei HC. Biofilm formation on dental restorative and implant materials. *J Dent Res.* 2010; 89:657–65. [PubMed: 20448246]
44. Kuramitsu HK, Wang BY. The whole is greater than the sum of its parts: dental plaque bacterial interactions can affect the virulence properties of cariogenic *Streptococcus mutans*. *Am J Dent.* 2011; 24:153–4. [PubMed: 21874934]
45. Hahnel S, Rosentritt M, Burgers R, Handel G. Surface properties and in vitro *Streptococcus mutans* adhesion to dental resin polymers. *J Mater Sci Mater Med.* 2008; 19:2619–27. [PubMed: 18197372]

46. Aykent F, Yondem I, Ozyesil AG, Gunal SK, Avunduk MC, Ozkan S. Effect of different finishing techniques for restorative materials on surface roughness and bacterial adhesion. *J Prosthet Dent*. 2010; 103:221–7. [PubMed: 20362765]
47. Bollen CM, Lambrechts P, Quirynen M. Comparison of surface roughness of oral hard materials to the threshold surface roughness for bacterial plaque retention: a review of the literature. *Dent Mater*. 1997; 13:258–69. [PubMed: 11696906]
48. Navarra CO, Cadenaro M, Codan B, Mazzoni A, Sergo V, De Stefano Dorigo E, Breschi L. Degree of conversion and interfacial nanoleakage expression of three one-step self-etch adhesives. *Eur J Oral Sci*. 2009; 117:463–9. [PubMed: 19627360]
49. Mei L, Busscher HJ, van der Mei HC, Ren Y. Influence of surface roughness on streptococcal adhesion forces to composite resins. *Dent Mater*. 2011; 27:770–8. [PubMed: 21524789]
50. Ferracane JL, Greener EH. The effect of resin formulation on the degree of conversion and mechanical properties of dental restorative resins. *J Biomed Mater Res*. 1986; 20:121–31. [PubMed: 3949822]
51. Cadenaro M, Antonioli F, Sauro S, Tay FR, Di Lenarda R, Prati C, Biasotto M, Contardo L, Breschi L. Degree of conversion and permeability of dental adhesives. *Eur J Oral Sci*. 2005; 113:525–30. [PubMed: 16324144]
52. Tay FR, Suh BI, Pashley DH, Prati C, Chuang SF, Li F. Factors contributing to the incompatibility between simplified-step adhesives and self-cured or dual-cured composites. Part II. Single-bottle, total-etch adhesive. *J Adhes Dent*. 2003; 5:91–105. [PubMed: 14964676]
53. Brambilla E, Gagliani M, Ionescu A, Fadini L, Garcia-Godoy F. The influence of light-curing time on the bacterial colonization of resin composite surfaces. *Dent Mater*. 2009; 25:1067–72. [PubMed: 19375790]
54. Bourbia M, Ma D, Cvitkovitch DG, Santerre JP, Finer Y. Cariogenic bacteria degrade dental resin composites and adhesives. *J Dent Res*. 2013; 92:989–994. [PubMed: 24026951]
55. Belli WA, Marquis RE. Adaptation of *Streptococcus mutans* and *Enterococcus hirae* to acid stress in continuous culture. *Appl Environ Microbiol*. 1991; 57:1134–1138.
56. Nascimento MM, Lemos JAC, Abranches J, Gonclaves R, Burne RA. Adaptive acid tolerance response of *Streptococcus mutans*. *J Bacterial*. 2004; 186:6383–6390.
57. Takahashi Y, Imazato S, Russell RR, Noiri Y, Ebisu S. Influence of resin monomers on growth of oral streptococci. *J Dent Res*. 2004; 83:302–6. [PubMed: 15044503]
58. Geurtsen W. Biocompatibility of resin-modified filling materials. *Crit Rev Oral Biol Med*. 2000; 11:333–55. [PubMed: 11021634]
59. Teughels W, Van Assche N, Sliepen I, Quirynen M. Effect of material characteristics and/or surface topography on biofilm development. *Crit Rev Oral Biol Med*. 2006; 17 (Suppl 2):68–81.
60. Kantorski KZ, Scotti R, Valandro LF, Bottino MA, Koga-Ito CY, Jorge AO. Surface roughness and bacterial adherence to resin composites and ceramics. *Oral Health Prev Dent*. 2009; 7:29–32. [PubMed: 19408812]
61. Khalichi P, Cvitkovitch DG, Santerre JP. Effect of composite resin biodegradation products on oral streptococcal growth. *Biomaterials*. 2004; 25:5467–72. [PubMed: 15142727]
62. Beyth N, Bahir R, Matalon S, Domb AJ, Weiss EI. *Streptococcus mutans* biofilm changes surface-topography of resin composites. *Dent Mater*. 2008; 24:732–6. [PubMed: 17897707]
63. Rinastiti M, Ozcan M, Siswomihardjo W, Busscher HJ, van der Mei HC. Effect of biofilm on the repair bond strengths of composites. *J Dent Res*. 2010; 89:1476–81. [PubMed: 20940354]
64. Hahnel S, Rosentritt M, Handel G, Burgers R. Influence of saliva substitute films on initial *Streptococcus mutans* adhesion to enamel and dental substrata. *J Dent*. 2008; 36:977–83. [PubMed: 18789569]

Figure 1. MTT assay result showing mean and standard error of the adherent *S. mutans* biomass growing on the surfaces of the five experimental resins blends.

Figure 2.
Quadratic relationship between biofilm formation (OD) and Hoy's δ_t of the resin blends.

Figure 3.

Live/dead staining of the biofilms adhering to the surface of experimental resin blends R₁-R₅. Living cells were green-stained, and the dead cells were red-stained. Sections corresponding to the resin surfaces were excluded from the corresponding stacks due to the auto-fluorescent signal present in most resins blends. In disk surfaces prepared from resin blends R₁-R₄, the biofilms were primarily alive, with areas covered by a multilayer of bacterial structures. In resin blend R₅, biofilms were characterized by sparse micro-colonies, almost completely occupied by dead cells. The major part of the resin surface derived from R₅ was free of bacterial colonization.

Figure 4.

SEM micrographs of biofilms adhering to the experimental resin blends R₁-R₅. The polymerized resin surfaces of R₁-R₄ were densely covered with biofilms consisting of several long cocci strings. The polymerized resin surfaces of R₅ contained thin biofilms, with localized regions that were not covered by bacterial cells (magnification 2000×).

Table 1

Composition of the experimental resins blends R₁₋₅ and Hoy's solubility parameters (δ).

Resin #	Neat Resin Composition	Hoy's solubility parameters (J/cm ³) ^{1/2}			
		δ_d	δ_p	δ_h	δ_t
R1	70 wt% E-BisADM 28.75 % TEGDMA	15.0	10.3	6.6	19.4
R2	70% BisGMA 28.75% TEGDMA	15.9	12.4	6.9	21.2
R3	70% BisGMA 28.75% HEMA	15.6	13.0	8.5	22.1
R4	40% BisGMA 30% TCDM 28.75% TEGDMA	16.5	12.9	7.0	22.1
R5	40% BisGMA 30% BisMP 28.75% HEMA	15.1	13.5	11.1	23.1

All resin blends contained 0.25 wt% camphorquinone and 1.0 wt% 2-ethyl-dimethyl-4-aminobenzoate. Abbreviations: E-BisADM = ethoxylated bisphenol A dimethacrylate; BisGMA = 2,2-bis[4-(2-hydroxy-3-methacryloylpropoxy)l-phenyl]propane; TEGDMA = triethyleneglycol dimethacrylate; HEMA = 2-hydroxyethyl methacrylate; TCDM = di(hydroxyethyl methacrylate)ester of 5-(2,5-dioxotetrahydrofurfuryl)-3-methyl-3-cyclohexane-1, 2'-dicarboxylic acid; BisMP = bis[2-(methacryloyloxy)ethyl]phosphate. δ_d = Hoy's solubility parameter for dispersive forces; δ_p = Hoy's solubility parameter for polar forces; δ_h = Hoy's solubility parameters for hydrogen bonding forces; δ_t = Hoy's solubility parameter for total cohesive forces, equivalent to Hildebrand's solubility parameter (d). $\delta_t = [(\delta_d)^2 + (\delta_p)^2 + (\delta_h)^2]^{1/2}$. All Hoy's parameter values are given in (J/cm³)^{1/2}.

All solubility parameters were calculated using a commercially available software (Computer Chemistry Consultancy <www.compchemconsul.com>).

Table 2

pH measurements obtained after adding distilled water, PBS or BHI to resins disks surfaces of R₁₋₅.

Solution	Resin	Measurement time				
		T=0h	T=3h	T=6h	T=12h	T=24h, after rinsing
H ₂ O	R1	6.61(0.03)a	6.77(0.14)a	6.73(0.30)a	6.67(0.04)a	6.60(0.04)a
	R2	6.62(0.04)a	6.31(0.09)b	6.41(0.30)a	6.53(0.03)a,b	6.62(0.04)a
	R3	6.66(0.02)a	6.35(0.07)b	6.42(0.36)a	6.43(0.02)b,c	6.61(0.03)a
	R4	6.07(0.01)b	6.06(0.04)b	5.85(0.34)b	6.32(0.05)c	6.55(0.04)a
	R5	3.23(0.14)c	3.15(0.40)c	3.05(0.22)c	3.10(0.20)d	4.40(0.07)b
PBS	R1	7.39(0.01)a,b	7.39(0.01)a	7.39(0.02)a	7.34(0.01)a	7.38(0.01)a
	R2	7.40(0.01)a	7.40(0.00)a	7.38(0.02)a	7.33(0.01)a	7.40(0.01)a
	R3	7.40(0.00)a	7.41(0.01)a	7.37(0.02)a	7.34(0.01)a	7.40(0.01)a
	R4	7.38(0.01)b	7.41(0.01)a	7.18(0.01)b	7.23(0.01)b	7.39(0.01)a
	R5	6.96(0.01)c	6.41(0.31)b	4.04(0.20)c	2.79(0.10)c	6.83(0.03)b
BHI	R1	7.07(0.01)a	7.10(0.01)a	7.12(0.01)a,b	7.15(0.01)a	7.08(0.01)a
	R2	7.07(0.00)a	7.11(0.00)a	7.12(0.01)a,b	7.16(0.01)a	7.10(0.01)a
	R3	7.07(0.01)a	7.11(0.00)a	7.13(0.01)a	7.16(0.00)a	7.10(0.01)a
	R4	7.07(0.00)a	7.11(0.00)a	7.11(0.01)b	7.13(0.02)a	7.09(0.01)a
	R5	6.90(0.02)b	6.90(0.07)b	6.02(0.03)c	4.10(0.12)b	6.83(0.03)b

The first set of measurements was performed immediately after the rinsing procedure. After that, measurements were performed after 3h, 6h, 12h and 24h. The last set of measurements was performed after discarding the solutions, rinsing the resin disks three times with water then reinserting new solutions inside the wells. Results are listed as means and standard deviations (SD). Similar letters indicate no statistical significance for $\alpha = 0.05$.