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Streptococcus mutans biofilm changes surface-topography of resin composites

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ABSTRACT

Objectives. Polymerized resin composites and nonpolymerized monomers are reported to accelerate bacterial growth. Furthermore, *in vivo*, resin composite restorations accumulate more plaque than other restorative materials. The purpose of this study was to test the hypothesis that bacteria–composite surface interaction causes changes in surface-topography.

Methods. Resin composite disks were polymerized between two glass slides. *Streptococcus mutans* cells were brought in contact with and grown on the disks for 1 day, 1 week or 1 month. The disks were analyzed using atomic force microscopy. One-month-aged composite specimens were assayed for changes in micro-hardness and bacterial outgrowth.

Results. Atomic force microscopy analysis revealed a time-dependent increase in root mean square (RMS) roughness ($p < 0.0001$). *S. mutans* outgrowth was accelerated following direct contact with the surface of aged composites, with no changes in micro-hardness.

Significance. Our results show that *S. mutans* growth on resin composite increases surface roughness without affecting micro-hardness. The change in surface integrity may further accelerate biofilm accumulation.

1. Introduction

Resin composite restorations tend to accumulate more dental plaque compared with other restorations [1–3]. It was reported that polymerization of resin composites is incomplete, as indicated by the low degree of conversion [4] and the finding that unpolymerized monomers can be extracted and used to accelerate the growth of cariogenic bacteria [5]. In addition, it was shown that polymerized resin composites accelerate *Streptococcus mutans* growth *in vitro* [6].

It is unclear whether bacterial growth is accelerated by the residual unpolymerized monomer only or by the polymerized resin as well. If the latter is true, it follows that polymerized resin composites will undergo surface changes as a result of biofilm–composite interaction. In the present study, surface properties of polymerized resin composites that had been exposed to bacterial biofilm were assessed. The following surface properties were measured: (i) surface roughness, using atomic force microscopy (AFM) (ii) micro-hardness and (iii) bacterial growth on aged composite, using the direct contact test (DCT).

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Table 1 – Materials

Material	Manufacturer	Lot number	Composition
Z250	3M ESPE, Dental St. Paul, MN USA	20020801	Filler: 66 vol% zirconia, silica Particle size: 0.01–3.5 μm Matrix: BIS-GMA, UDMA, BIS-EMA
Tetric Ceram	Ivoclar Vivadent Ltd., Liechtenstein	C30477	Filler: 60 wt% barium glass, ytterbium trifluoride Ba-Al-fluorosilicate glass, silicon dioxide, spheroid mixed oxide Particle size: 0.04–3.0 μm Matrix: BIS-GMA, UDMA, TEGDMA
Heliomolar	Ivoclar Vivadent Ltd.	E34235	Filler: 64 vol% silicon dioxide, ytterbium III, fluoride Particle size: 0.04–0.2 μm Matrix: BIS-GMA, UDMA, D_3MA

BIS-GMA, bisphenol-A-glycidyldimethacrylate; UDMA, urethane dimethacrylate; BIS-EMA, bisphenol-A-ethoxy dimethacrylate; D_3MA, decamethacrylate; TEGDMA, triethleneglycoldimethacrylate.

2. Materials and methods

2.1. *Bacteria and growth conditions*

S. mutans (ATCC# 27351) was cultured overnight at 37 °C in brain–heart infusion (BHI) broth (Difco, Detroit, MI, USA) supplemented with bacitracin (Sigma, St. Louis, MO, USA) (0.0625 g/mL). The top 4 mL of the suspension were harvested into a fresh test tube and centrifuged for 10 min at 3175 × *g* to isolate bacteria in the mid-exponential phase. The supernatant was discarded and the bacteria were resuspended in 5 mL of phosphate-buffered saline (PBS) (Sigma) containing 0.0625 g/mL of bacitracin. A 800 μL volume of the culture was diluted to 10^6 cells/mL.

2.2. *Tested materials*

Commercial resin composites were tested for surface changes following the growth of biofilm on their surface. The materials used were: Z250 (3M ESPE Dental St. Paul, MN, USA), Tetric Ceram (Ivoclar Vivadent, Liechtenstein) and Heliomolar (Ivoclar Vivadent) (Table 1).

2.3. *Atomic force microscopy*

An equal amount of material was pressed between 2 glass slides, forming 45 similar disks, 15 of each resin composite brand (5 ± 1 mm diameter, 1 mm thickness). The disks were photo-polymerized for 40 s on each side, using a conventional light-curing unit (Elipar high light, 3M ESPE Dental) with a light intensity of 600 ± 3 mW/cm^2. To immobilize the bacteria and obtain direct contact with the resin composite, a 10 μL drop of bacterial suspension was placed on each of 15 disks, 5 disks from each brand, which were then incubated at 37 °C for 1 h to evaporate excess water. The disks were then placed in tubes containing 5 mL of BHI broth supplemented with 0.0625 g/mL of bacitracin and incubated at 37 °C for 24 h to allow the formation of 1-day-old biofilm. A similar procedure was performed on an additional 30 disks to form 1-week and 1-month-old biofilms. In the latter two groups, the broth was replaced every 48 h. Before replacing the broth, the disks were vortexed for 10 s and the broth was decanted without disturbing the biofilm. The control groups comprised 15 additional disks from each brand, 5 of which were incubated in sterile broth for 1 day, 1 week or 1 month without a bacterial inoculum. Lack of contamination was verified by microscopic examination.

At the end of the test period, the disks were washed 3 times for 30 s with double-distilled water (DDW) and placed in test tubes containing 5 mL of DDW supplemented with 0.02% azide, at 4 °C. To obtain a clean surface without bacteria or bacterial products, the disks were sonicated for 15 min (Tuttnauar-Ultrasonic cleaner, model U1424 43 KHz, Tuttnauer, Israel). The disks were then dried at room temperature (20 °C). Bradford Protein staining (Bio-Rad Protein Assay, BIO-RAD Laboratories GmbH, Munchen, Germany) was performed on each disk to ensure that all biofilm residues had been removed [7].

Each disk was scanned by an atomic force microscope (AFM) in three different areas, to obtain 15 measurements of root mean square (RMS) roughness for each test group. A MultiMode™ SPM scanning probe microscope (Digital Instruments, Santa Barbara, CA, USA) using nanoprobe etched silicon cantilevers with a spring constant of $k = 0.06$ N/m in TappingMode™ AFM, was used to record the resin composite surface changes and measure the surface root mean square (RMS) roughness by oscillating the cantilever in free air at its resonant frequency.

2.4. *Micro-hardness test*

For the micro-hardness measurements, seven disks were made from each of the resin composite materials, using a Teflon mold (6 mm in diameter, 3 mm thickness). The disks were light-polymerized as described above and then processed as described for the AFM 1 month group. Seven additional disks for each resin composite kept under sterile conditions served as control.

The disks were cold mounted in CITO FIX (Struers, Rødone, Denmark). Vicker's hardness measurements were obtained using a 0.98 N load. The appropriate load was applied for 10 s and the indentation size was recorded 10 s later. A Vickers diamond tip was used to make indentations in three positions on each specimen. Under an optical microscope, each

indentation was measured diagonally from one edge of the diamond shaped impression to the other edge, across each of the diagonals. The data were averaged to provide the mean diagonal length for each material and the Vickers hardness value associated with each material was calculated.

2.5. Bacterial growth on aged composite

Bacterial growth was measured using the direct contact test [6,8]. In brief, the sidewalls of 8 wells in a 96-well flat bottom microtiter plate (Nunclon, Nunc, Copenhagen, Denmark) were coated evenly with an equal amount of the same resin composite (30 ± 5 mg/well). The materials were light polymerized as above. For material aging, 250 μL of PBS were added to each well, and the plate was then incubated at 37 °C. The PBS was replaced every 48 h for a period of 1 month, whereupon the plate was air-dried. A 10 μL volume of the *S. mutans* suspension was placed on the resin composite surface, and the plates were incubated for 1 h at 37 °C ensuring direct contact between the bacteria and the tested surface. Eight wells in the same microtiter plate, lacking the resin composites, served as control. The plate was then placed horizontally and 220 μL of BHI broth supplemented with 0.0625 g/mL of bacitracin were added to each well. The plate was incubated in a temperature-controlled microplate spectrophotometer (VERSAmax, Molecular Devices Corporation, Menlo Oaks Corporate Center, Menlo Park, CA, USA) set at 37 °C, with 5 s mixing before each reading. The growth of the bacteria shed from the biofilm was assessed by following the changes in absorbance (OD_{650}) every 20 min for 16 h. The data were plotted, providing bacterial growth curves for each well. Bacterial growth on aged composite was repeated five times.

2.6. Statistical analysis

The micro-hardness results and the RMS roughness ratios of the surfaces were analyzed in a model of repeated measures, using multivariate tests (Pillai's Trace, Wilks' Lambda, Hotelling's Trace and Roy's Largest Root) at a significance level of $p < 0.01$. Turbidity experiments were analyzed by comparing the slopes of the growth curves. The linear portion of the logarithmic growth curve was analyzed by one-way ANOVA and the Tukey multiple comparison score. The level of significance was set at $p < 0.05$.

3. Results

3.1. Atomic force microscopy

Topographic changes that occurred in all the test materials are shown in the three-dimensional surface views of Heliomolar without biofilm as compared with 1 month Heliomolar with biofilm (Fig. 1a and b). AFM analysis of Heliomolar, Z250 and Tetric Ceram, after bacteria–surface interaction during the three different time periods is shown in Fig. 2. One-week-old *S. mutans* biofilm on Heliomolar disks increased significantly, the roughness compared with that of similar disks kept in non-inoculated sterile broth ($p < 0.0001$). An increase in RMS roughness was evident after 1 month in all the test materials

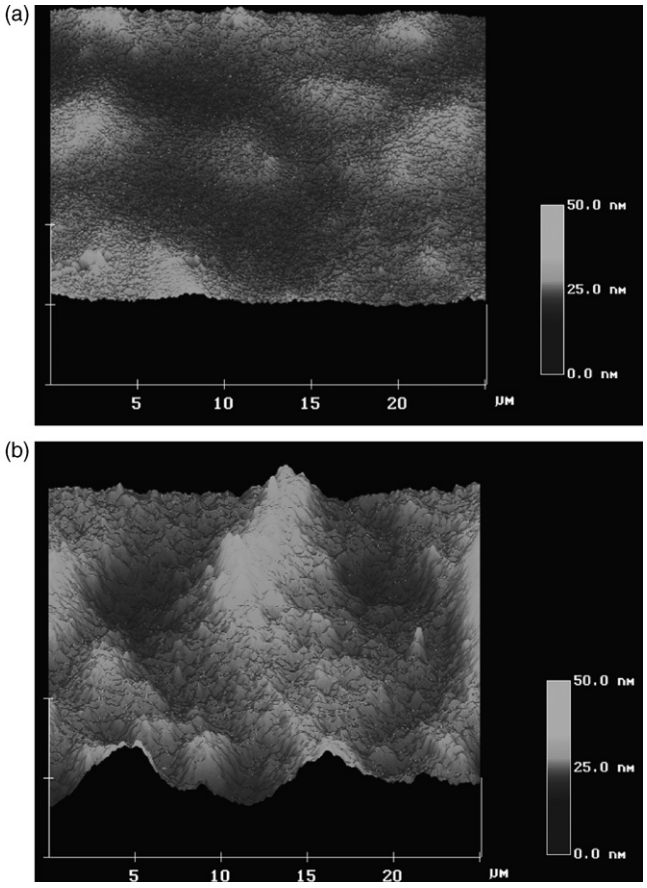

Fig. 1 – Atomic force microscope image (25 μm × 25 μm) of Heliomolar surface following: (a) 1-month Heliomolar surface without bacteria and (b) 1-month bacteria–resin composite interaction.

compared with that of the control groups ($p < 0.01$). Of the three resin composites, the Heliomolar group showed the highest roughness ratio ($p < 0.0001$). Bradford Protein staining applied to the surface of the test and control disks showed no color change, indicating all proteins had been removed.

3.2. Micro-hardness test

The micro-hardness results showed no significant difference ($p > 0.05$) after 1 month of bacteria–surface interaction compared with that of the control groups in all the three resin composites (data not shown).

3.3. Bacterial growth on aged composite

S. mutans growth following bacteria–material interaction determined by turbidity measurements in a 96-well microtiter plate is shown in Fig. 3. Each point on the curves represents the mean value measured in eight wells containing the same test material following bacteria–surface interaction. In all the three 1-month-aged resin composites, the logarithmic growth rate was increased compared with that of the control group in which the bacterial suspension was placed on the polystyrene microtiter well wall ($p < 0.05$). Of the

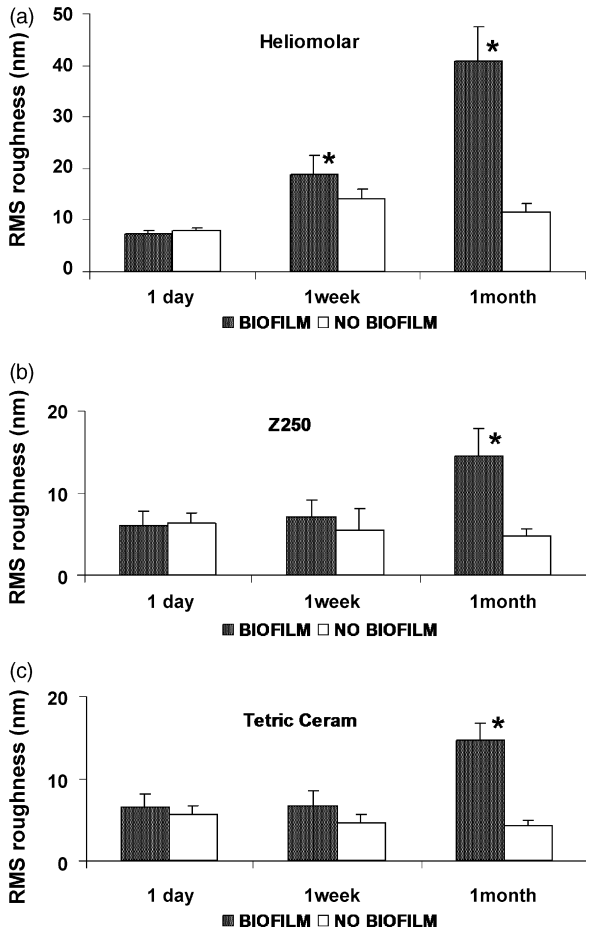

Fig. 2 – Atomic force microscope root mean square (RMS) roughness analysis after 1 day, 1 week and 1 month of bacteria–surface interaction in three resin composites compared with that of similarly prepared controls with no bacteria. The values were significantly higher ($^{*}p < 0.0001$) compared with the control after 1 week in Heliomolar and after 1 month in all three tested materials: (a) Heliomolar, (b) Z250 and (c) Tetric ceram.

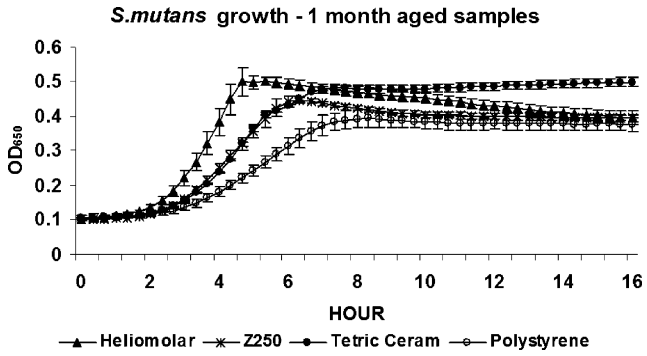

Fig. 3 – Turbidity measurements of *S. mutans* outgrowth following bacteria–resin composite direct contact. Interaction of three 1-month-aged resin composites compared with the control (polystyrene). The outgrowth of the shed bacteria from the biofilm was measured every 20 min for 16 h. Each point on the curve is the mean optical density (OD_{650}) measured in seven wells similarly prepared in the same microtiter plate. All the three aged resin composites increased ($p < 0.05$) the *S. mutans* logarithmic growth rate following bacteria–surface interaction compared with that of the control group.

three materials, Heliomolar showed the highest growth rate ($p < 0.05$).

4. Discussion

4.1. *Atomic force microscopy*

Surface roughness promotes bacterial adhesion and plaque retention [9,10], which may influence the longevity of the restoration. We used AFM to measure the surface-roughness modifications following bacteria–composite interaction. The significant increase ($p < 0.0001$) in surface roughness was time-dependent. This finding cannot be attributed to biomass increase on the surface as previously proposed [11], since (i) there was no evidence for the presence of proteins on the surface of the tested composite samples, (ii) the increase in RMS roughness was in the range of 40 nm, far lower than the size of a possible residual bacterium and (iii) there were no differences in the micro-hardness of the various samples which is measured in the micrometer range. It is conceivable that partial surface degradation was due to the bacteria–material interaction.

4.2. *Bacterial growth on aged composite*

Various studies investigated the antibacterial properties of commercial resin composites and their constituents [12–14]. Following previous reports that polymerization of resin composites is incomplete [4] and that the composite extracts as well as monomers stimulate growth of caries-associated bacteria [5], we aged the polymerized resin composite samples for 1 month in PBS to allow primary elution of the monomers and only then tested their effect on bacterial growth. Enhanced bacterial growth was detected after direct contact of the bacteria with the surface of polymerized resin composites. These results coincide with previously reported *in vivo* findings [2]. Assuming that the vast majority of the unpolymerized monomer was eluted during the aging period, it is unlikely that the enhanced bacterial growth is attributable solely to the residual monomers.

Little is known about the mutual effect of bacteria and the surfaces they colonize, especially the surfaces of dental restorations. Unlike previous studies, we investigated not only the parameters related to bacterial growth and activity but also the parameters related to surface qualities of resin composites. The data reported here add to our understanding of the clinical performance of resin composite restorations. In the long run, resin composite restorations suffer from degradation owing to oral environmental conditions and to their physical and chemical properties. Part of the problem is the attachment and adhesion of microorganisms to the sur-

face, as well as biofilm formation. Secondary caries is the most frequent cause for the replacement of resin composite restorative materials [15,16]. In the present study, we examined the material–bacteria mutual effect. The nanometric surface changes caused by bacteria, increase surface roughness, which in turn, encourages more bacteria to attach to the resin composite and colonize. With time the roughness continues to increase. This paradigm is a "vicious cycle" caused by the bacteria–surface interaction, which may cause restoration failure.

Bacteria invading the interface between the tooth and the restorative material are the principal etiologic factor responsible for secondary caries [17]. Furthermore, the gingival margin of class II resin composite restorations is the *locus minoris resistencia*, due to the fact that (i) this margin is a resin-rich area since it cannot be polished, (ii) this area is not easily cleaned and is thus prone to continuous biofilm accumulation [18] and (iii) it is susceptible to microleakage and biodegradation associated with salivary esterase activity [19].

Thus we conclude, resin composites inherently enhance bacterial growth, undergo degradation and are prone to compromised longevity. Further studies are indicated to develop long-lasting antibacterial surface properties in order to inhibit unfavorable bacteria–surface interactions.

5. Conclusion

The null hypothesis was partly confirmed, the results showing that *S. mutans* growth on resin composites increases surface roughness without affecting micro-hardness. This change in surface integrity may further increase biofilm accumulation.

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