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Influence of Light-curing Parameters on Biofilm Development and Flexural Strength of a Silorane-based Composite

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Clinical Relevance

Silorane-based composite is less prone to *Streptococcus mutans* biofilm development compared with a conventional methacrylate-based composite and has an acceptable flexural strength. Lower biofilm development may reduce resin composite superficial stain and recurrent caries, thus improving the longevity of restorations.

SUMMARY

Objectives: The aim of this study was to evaluate the differences in biological and mechanical performances of a silorane-based and a methacrylate-based composite. Another aim was to assess the influence of light-curing time and light-curing intensity on *in vitro* biofilm formation and flexural strength of the two tested composites.

Methods: Experiment 1: 432 specimens obtained from a silorane-based composite and from a standard methacrylate-based composite were divided into six groups and light-cured for 10, 20, 30, 40, 60, or 80 seconds, using one of two light-curing intensities, 400 mW/cm^2 or 800 mW/cm^2. At 24 hours, a monospecific *Streptococcus mutans* biofilm adherent to the surfaces of the samples was obtained. Then, a colorimetric technique (MTT assay) was used to evaluate the adherent viable biomass. Two samples per group were observed using confocal laser scanning microscopy. Analysis of variance (ANOVA) and Tukey tests were used to analyze the results ($p<0.05$). Experiment 2: 192 bar-shaped specimens were obtained and light-cured as in the previous experiment. A three-point bend test using a universal testing machine was performed to obtain flexural strength values. ANOVA and Tukey tests were used to analyze the results ($p<0.05$).

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Results: In experiment 1, a highly significant difference ($p<0.0001$) in biofilm development was shown between silorane-based and methacrylate-based composites. In fact, the silorane-based composite exhibited better biological performance. Significant differences were also found between the two light-curing intensities ($p<0.018$) and for curing times ($p<0.0001$): silorane-based composite light-cured for 80 seconds at 800 mW/cm^2 light-curing intensity showed the lowest biofilm development. In experiment 2, a significant difference in flexural strength ($p<0.0318$) was only found between the different composites. Nevertheless, both resin composites showed flexural strength values in accordance with International Organization for Standardization guidelines even after 10 seconds of light-curing time.

Conclusions: Silorane-based composite was less prone to biofilm development compared with a methacrylate-based composite. Acceptable flexural strength values for both composites were obtained after 10 seconds of light-curing time.

INTRODUCTION

Thanks to their characteristics,[1] resin-based composites (RBCs) have become the most used materials in restorative dentistry.[2] RBCs are generally cured by light-induced polymerization of monomers. Nevertheless, because complete polymerization of these materials never occurs,[3-6] monomers may leach out of composites.[7-10] Previous data showed that the light-curing time of an RBC is a crucial factor in determining the characteristics of surface colonization.[11] This is a very important aspect as it leads to biofilm development, which is one of the most important factors in caries formation.[12-14] In fact, an imbalance of the oral microbial communities combined with an increase of cariogenic bacteria is considered the first step in primary and secondary caries development.[15-18] *Streptococcus mutans* is the main microorganism responsible for caries lesions, hence influencing restoration success over time.[14,19-21]

Another issue with RBC materials is polymerization shrinkage, which is caused by the conversion of monomer molecules into a polymer network.[22] This process induces stresses into the resin restorations and the surrounding tooth structure, which leads to microfractures and/or blistering and, eventually loss of marginal seal.[23-27] Two strategies have been used to overcome this drawback: lowering the number of reactive sites per volume and using new resins.[28] Increasing the molecular weight of the monomers and the filler load are two methods to reduce the number of reactive sites, but an augmented molecular weight can compromise the handling characteristics of resin composites and increase polymerization stress, whereas an overload of inorganic filler saturates the resin capacity to incorporate its particles.[28]

Since Bowen[29] introduced methacrylate-based chemistry in dentistry in 1965, different alternatives have been studied, some of them by Bowen himself. Research on epoxy resins has led to the development of a new kind of monomer, the siloranes.[30-32] A silorane monomer has a hybrid molecule made of a central siloxane ring to which oxirane structural moieties are attached. The silorane matrix is formed by silorane monomers through a cationic ring-opening polymerization process. The opening of the epoxide rings compensates for the polymerization shrinkage,[30] thus generating a material that possibly overcomes one of the main problems of modern RBCs. Moreover, it has been suggested that the siloxane backbone of siloranes provides hydrophobic characteristics to these restorative materials.[28]

Compared with methacrylate-based restorative materials, silorane-based composites show very low polymerization shrinkage but overall have mixed mechanical performances. The silorane-based material has relatively higher flexural strength/modulus and fracture toughness but lower compressive strength and microhardness than the methacrylate-based composites.[33,34]

So far, no studies have investigated biofilm development and flexural strength of silorane-based composites as a function of their curing parameters. Therefore, the aim of this study was to assess the existence of differences in biological (*S. mutans* biofilm formation) and mechanical (flexural strength) performances of a silorane-based and a methacrylate-based composite. Another aim was to assess the influence of light-curing time and light-curing intensity on *in vitro* biofilm formation and flexural strength of the two tested composites. The tested null hypothesis was that the silorane-based and the methacrylate-based composites would not show differences in *S. mutans* biofilm formation and flexural strength. The second null hypothesis was that light-curing time and light-curing intensity would not have any influence on the two tested materials in terms of *S. mutans* biofilm formation and flexural strength.

Table 1: *Composite Resin Compositions According to Manufacturer*

Composite	Organic matrix	Filler
Filtek Silorane	Silorane (3,4-epoxycyclohexylethylcyclopolymethylsiloxane,bis-3,4-epoxycyclohexylethylphenylmethylsilane)	Silanized quartz, yttrium fluoride
Z250	Bis-GMA, UDMA, Bis-EMA	Zirconia/silica

Abbreviations: Bis-GMA, bisphenol-A-glycidyl methacrylate; UDMA, Urethane dimethacrylate Bis-EMA, bisphenol-A-ethoxy dimethacrylate.

METHODS AND MATERIALS

Two commercially available RBCs, based on either silorane (Filtek Silorane, 3M ESPE, Seefeld, Germany) or methacrylate-based resin chemistry (Filtek Z250, 3M ESPE) were used in this study. The shade used was A3, and the compositions are shown in Table 1. All reagents and multi-well plates used in the present study were obtained from Sigma-Aldrich (St Louis, MO, USA) unless otherwise specified.

Specimen Preparation for the Microbiological Procedures

The wells of a 96-well polystyrene plate were separated from the base of the plate and used as molds to create standardized test disks (6.4mm diameter and 1.5mm thickness). For the preparation of a single RBC test specimen, an excess amount of uncured resin-based composite was placed in a single trimmed well, covered with a Mylar strip to prevent the formation of an oxygen-inhibited layer, and then condensed against a glass plate. The disks were randomly divided into six groups and light-cured for 10, 20, 30, 40, 60, and 80 seconds using a light-curing unit (Spectrum 800, Dentsply International Inc, York, PA, USA). The light-curing unit was set at two light-curing intensities (400 and 800 mW/cm^2), thus generating two subgroups differing in light-curing intensity for each time group. The light-guide end was placed directly in contact with one of the two Mylar strips covering the composite surface. A total of 18 disks were produced for each curing time group and light-curing intensity subgroup. After polymerization the specimens were carefully removed from the wells and checked for visible surface irregularities. No finishing procedure was adopted. The plates were stored in a dark place for 24 hours at 37°C to allow complete polymerization of the disks. Then, 200 μL of sterile phosphate buffered saline (PBS) was placed in each well, and the plates were stored for an additional 7 days to allow for the leaching of most of the residual monomers. To remove the leached monomers, each well was washed twice every day using sterile PBS. Subsequently, 16 disks for each group were transferred to new 96-well polystyrene plates. These plates were then sterilized using a chemiclave with hydrogen peroxide gas plasma technology (Sterrad, ASP, Irvine, CA, USA). By limiting the maximum temperature to 45°C, heat-related damage of the RBC specimens was avoided.

Bacteria

All the culture media were obtained from Becton-Dickinson (BD Diagnostics-Difco, Franklin Lakes, NJ, USA). A pure suspension of *S. mutans* strain ATCC 35668 in brain-heart infusion broth (BHI) was obtained after 12 hours of incubation at 37°C in a 5% supplemented CO_2 environment. Cells were harvested by centrifugation (2200*g*, 19°C, 5 minutes), washed twice with sterile PBS, and resuspended in the same buffer. The cell suspension was subsequently subjected to low-intensity ultrasonic energy in order to disperse bacterial chains, and the optical density (OD) was adjusted to 0.3 OD units at 550 nm (Genesys 10-S, Thermo Spectronic, Rochester, NY, USA), which corresponds to a microbial concentration of 3.65×10^8 cells/mL.

MTT Assay Reagents

A tetrazolium salt (MTT) stock solution was prepared by dissolving 5 mg/mL of thiazolyl blue tetrazolium bromide [3-(4,5-)-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] in sterile PBS, and a phenazinium salt (PMS) stock solution was prepared by dissolving 0.3 mg/mL of N-methylphenazinium methyl sulphate in sterile PBS. The solutions were stored at 2°C in light-proof vials until the day of the experiment, when a fresh measurement solution was made by mixing 1 mL of MTT stock solution, 1 mL of PMS stock solution, and 8 mL of sterile PBS.

A lysing solution was prepared by dissolving 10% vol/vol of sodium dodecyl sulphate and 50% vol/vol of dimethylformamide in distilled water.

Saliva Collection

Unstimulated saliva from three healthy donors was used in this study according to Guggenheim and others.[35] Saliva was collected in chilled test tubes,

pooled, heated at 60°C for 30 minutes to inactivate endogenous enzymes, and then centrifuged (12.000*g*) for 15 minutes at 4°C. The supernatant was transferred in sterile 10-mL tubes, then stored at −20°C. Immediately before starting the experimental session, saliva was thawed at 37°C for 1 hour. Next, 100 μL of saliva was placed into each well of the specimen-containing sterilized plates, and the plates were incubated for 4 hours at 37°C. Then, the saliva was blotted out and the wells were gently rinsed twice with 200 μL of sterile PBS.

Biofilm Development

In this step, 20 μL of the bacterial suspension in early log phase and 180 μL of sterile BHI were placed in each well. The plates were incubated for 24 hours at 37°C in a 5% supplemented CO_2 environment to allow biofilm development. The culture was then discarded and the wells were carefully washed twice with sterile PBS to remove nonadherent cells.

MTT Assay

Specimen-containing plates were filled with 100 μL of MTT solution for each well; the plates were incubated for 3 hours in a dark place at 37°C: during incubation, microbial redox systems converted the yellow salt to intracellular insoluble purple formazan. Then MTT solution was gently discarded, and the intracellular formazan crystals were dissolved by adding 100 μL of lysing solution to each well and incubating again for 1 hour at room temperature in a dark place. Finally, 90 μL of suspension was taken from each well and its absorbance was measured with a spectrophotometer at 550 nm (Genesys 10-S) and expressed as OD units.

Confocal Laser Scanning Microscopy

Two disks for each experimental group were prepared for confocal laser scanning microscopy (CLSM) analysis. However, because of the number of specimen groups, it was decided to analyze only the 10- and 80-second curing time groups at 400 mW/cm^2 curing intensity. This decision was taken after the MTT results highlighted a major difference in biofilm development between these curing time groups.

After the 24-hour incubation, the biofilm growing on the disks was gently washed with PBS to remove nonadherent cells and stained using the FilmTracer Live/Dead Biofilm Viability Kit for microscopy (Invitrogen Ltd, Paisley, UK). The fluorescence from stained cells adherent to the samples was observed using a CLSM (Leica TCS SP2, Leica Microsystems, Wetzlar, Germany). Four randomly selected image stack sections were recorded for each biofilm specimen. Confocal images were obtained using a dry 20× with numerical aperture (NA) of 0.7 objective and digitalized using the Leica Application Suite Advanced Fluorescence Software (LAS AF, Leica Microsystems) at a resolution of 1024 × 1024 pixels, with a zoom factor of 1.0. For each image stack section an average intensity projection and a three-dimensional (3D) reconstruction were obtained. The average intensity projections were done using ImageJ (National Institutes of Health, Bethesda, MD, USA), and Drishti (Ajay Limaye, Australian National University, CAN, AUS http://sf.anu.edu.au/Vizlab/drishti/) was used for 3D reconstructions.

Specimen Preparation and Flexural Strength Evaluation

A modified procedure from the International Organization for Standardization (ISO) 4049/2009 guidelines was used for the flexural strength evaluation. Briefly, a 2-mm thick polyether strip (Impregum, 3M ESPE AG) was obtained; the strip was then cut to obtain multiple bar-shaped standardized holes with a length of 10 mm and a width of 2 mm. For the preparation of RBC test specimens, an excess amount of uncured RBC was placed in the standardized holes, covered with Mylar strips to prevent the formation of an oxygen-inhibited layer, and then condensed against a glass plate to remove excess material. The glass plate was then removed, and the bars from each tested RBC were randomly divided into six groups and light-cured for 10, 20, 30, 40, 60, or 80 seconds using a light-curing unit (Spectrum 800). The light-curing unit was set at two light-curing intensities (400 or 800 mW/cm^2), thus generating two subgroups differing in the light-curing intensities for each time group. The light-guide end was placed directly in contact with one of the two Mylar strips covering the RBC surface. Eight bars for each light-curing time and light-curing intensity subgroup were produced. After polymerization, the specimens were carefully removed from the strip, checked for visible surface irregularities, and stored in a dark place for 24 hours at 37°C to allow complete polymerization.

After that, the bars were submitted to a three-point bend test with a universal testing machine with a crosshead speed of 1 mm/min. The maximum loads at fracture were obtained and the flexural

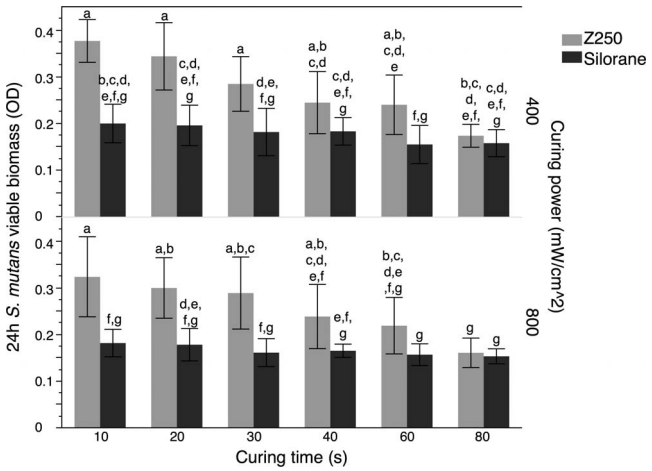

Figure 1. *Biofilm development expressed as OD. Bars represent means and error bars represent standard deviation.*

strength (σ) was calculated in megaPascals (MPa) by using the following formula: $\sigma = 3FL/(2BH^2)$, where F is the maximum load (in Newtons), L is the distance between the supports (in millimeters), B is the width of the specimen (in millimeters), and H is the height (also in millimeters). The formula was solved assuming that the prepared bars had a 2 mm width and a 2 mm thickness, and the custom-made support for the bars had a distance (L) equal to 8 mm.

Statistical Analysis

All statistical analyses were performed using statistical software (JMP 10.0, SAS Institute, Inc, Cary, NC, USA). The OD and flexural data are reported throughout the text as means and standard deviations (SDs) calculated from the natural values.

Data were analyzed by three-way analysis of variance (ANOVA) with balanced data in which light-curing time (six levels, ie, 10, 20, 30, 40, 60, or 80 seconds), light-curing intensity (two levels, ie, 400 or 800 mW/cm^2), and resin composite type (two levels, ie, methacrylate-based or silorane-based composites) were fixed factors. Homogeneity of variances was preliminarily checked using Bartlett's test. Tukey's post hoc test was used to highlight significant differences ($p<0.05$).

RESULTS

Experiment 1

Biofilm development on resin composite discs, expressed as mean OD ± 1 SD, is reported in Figure 1. Methacrylate-based and silorane-based composites showed significantly different biofilm development ($p<0.0001$). Light-curing time ($p<0.0001$) and light-curing intensity ($p<0.0108$) were also found to be significant factors in influencing biofilm development.

For each light-curing time and light-curing intensity group, except 80 seconds, silorane-based composite demonstrated lower biofilm development compared with the methacrylate-based composite. Extended light-curing times and higher light-curing intensities showed a reduction in OD values for both resin composites. However, this phenomenon proved to be significant only for the methacrylate-based resin composite.

The lowest biofilm development was obtained on the surfaces of silorane-based composite light-cured for 80 seconds at 800 mW/cm^2 light-curing intensity, whereas the highest biofilm development was obtained with methacrylate-based composite light-cured for 10 seconds at 400 mW/cm^2 light-curing intensity.

As shown in Figure 2, the methacrylate-based composite light-cured for 80 seconds at 400 mW/cm^2 light-curing intensity and silorane-based composite light-cured for 10 seconds and for 80 seconds at 400 mW/cm^2 light-curing intensity resulted in similar biofilm development with several live (green) and dead (red) *S. mutans* colonies covering the surface of the samples. In contrast, the methacrylate-based composite light-cured for 10 seconds at 400 mW/cm^2 light-curing intensity showed increased biofilm development; most of the surface was covered by live (green) *S. mutans* colonies.

Experiment 2

Flexural strength expressed as mean MPa ± 1 SD is shown in Figure 3**.** Three-way ANOVA did not show any interaction among the considered factors; therefore, analysis was performed for each factor according to a one-way ANOVA model. Flexural strength was only influenced by composite type ($p<0.0318$), and methacrylate-based composite showed higher flexural strength than silorane-based composite. In particular, the best results were obtained by methacrylate-based composite at 80 seconds of light-curing time and 800 mW/cm^2 light-curing intensity; however, they were not significantly different from the other methacrylate-based composite subgroups. Considering silorane-based composite, the best results were obtained for the 80 second 400 mW/cm^2 group. No significant differences between the other silorane-based composite subgroups were observed.

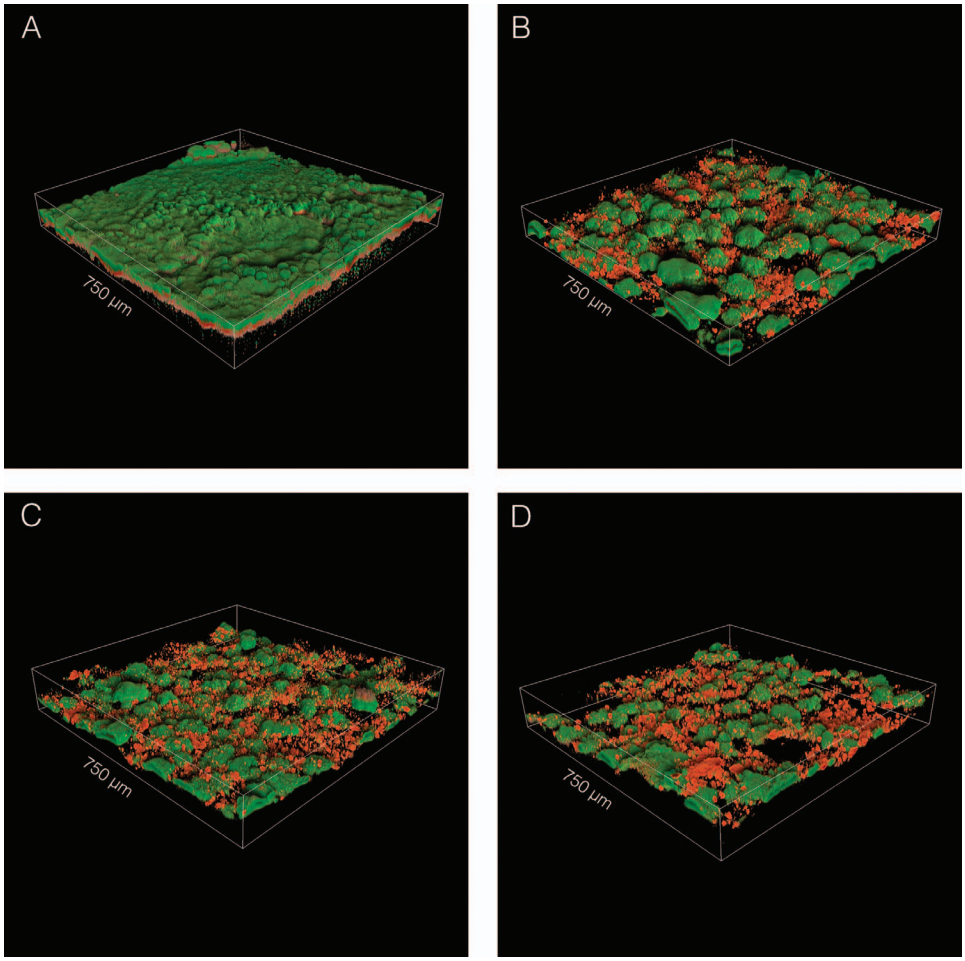

Figure 2. *CLSM 3D reconstructions of the biofilms, which were stained with live/dead stain. Green represents live bacteria and red represents non-viable, dead bacterial cells. Letters refer to the different experimental groups: A = 10 seconds 400 mW/cm^2 methacrylated-based composite, B = 80 seconds 400 mW/cm^2 methacrylated-based composite, C = 10 seconds 400 mW/cm^2 silorane-based composite, D = 80 seconds 400 mW/cm^2 silorane-based composite.*

DISCUSSION

Silorane-based composites were introduced as alternatives to conventional methacrylate-based composites to reduce polymerization shrinkage.[7-10] As bacterial colonization is an important factor for restoration longevity, the evaluation of the biological properties of these alternative resin composites seems to be another important issue to be investigated.

The best way to obtain data on bacterial colonization of the composite surface is to use an *in vitro* experimental model.[36-39] In this study, a Drip-flow reactor was chosen to achieve similar growth conditions for all resin specimens and to keep all the experimental parameters under controlled conditions.[37] Besides the experimental setup of the reactor, different parameters related to the material characteristics need to be considered, in particular the surface roughness and the curing process parameters. The influence of surface roughness on biofilm development was excluded by polymerizing the specimens against a Mylar strip. This technique allowed obtaining specimens with a mean surface roughness of $R_a = 0.06$ µm (data not shown), which is below the 0.2-µm threshold introduced by Bollen and others[40] in the 1990s. The results of this study

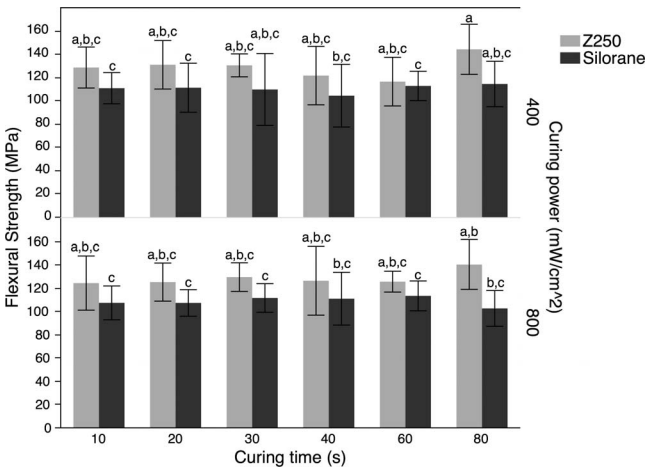

Figure 3. *Flexural strength expressed as megaPascals. Bars represent means and error bars represent standard deviations.*

suggested that R_a values below the specified threshold do not have a significant influence on biofilm development. Regarding the curing process, it has been demonstrated that light-curing time and light-curing intensity deeply influence biofilm development on resin composite surfaces.[11] Consequently it was decided to light-cure the specimens at six different times using two light-curing intensities to obtain data on the influence of these variables. While not of clinical relevance, 80 seconds of light-curing time was used in order to have a group of specimens with the highest degree of conversion possible.

The results of the study allow the rejection of the first null hypothesis because the silorane-based composite surfaces showed a reduction in biofilm development compared with the methacrylate-based composite ($p<0.0001$). In particular, the methacrylate-based resin composite showed a decreasing colonization trend as light-curing time increased, which agrees with the results of a previous study.[11] In contrast, silorane-based composite did not present significantly different values among light-curing times tested. These results allowed us to suppose that physicochemical properties, such as surface roughness and hydrophobicity, could influence material biological behavior. Given that in this study the influence of surface roughness variation could be excluded, it is possible to suggest that silorane had increased hydrophobicity, which makes this material less susceptible to biofilm development.

As for light-curing time, a statistically significant difference ($p<0.018$) in biofilm development was found between the two tested light-curing intensities, but only in methacrylate-based composite. This suggests that for these materials a better biological performance can be achieved by using the highest light-curing intensity tested (800 mW/cm^2).

The results of this study allowed a better comprehension of the tendency of silorane-based composites to develop biofilm, as no other authors have worked on this topic. However, a previous article by Buergers and others[31] demonstrated that the susceptibility of silorane-based composites to bacterial adhesion *in vitro* is lower than for four conventional methacrylate-based composites. The article suggested that the matrix of silorane-based composite, in particular its hydrophobicity, may negatively influence bacterial adhesion,[31] thus confirming our hypothesis. However, saliva was not used, as it was stated that the protocol was kept as simple as possible and that saliva was not the only factor differentiating an *in vitro* study from an *in vivo* study. Yet, another *in vitro* work did not show differences in bacterial adhesion between silorane-based and methacrylate-based composites. Nevertheless, surface roughness values of composites were too nonhomogeneous to easily compare the different groups.[41] Up to now only two *in situ* studies evaluated the biological performances of silorane-based composite. In the first study, Claro-Pereira and others[42] showed similar adhesion values for silorane-based and methacrylate-based composites. Nevertheless, the presence of several variables that were difficult to control and the limited number of subjects involved are weak points of this work. Instead, another *in situ* study evaluated the demineralization of dentin next to multiple restorative materials.[43] Results highlighted a high dentin demineralization associated with silorane-based composite. However, it is difficult to understand how restorative materials without the incorporation of any antibacterial principle can influence dentin demineralization in their proximity. Moreover, in this study, specimens were kept in a prosthesis full of acrylic resin, in which the oral flora is probably very different from the one present on teeth surfaces. With regard to clinical behavior, three clinical trials failed to highlight differences in clinical behavior between methacrylate-based and silorane-based composites.[44-46] In these studies no restoration failed for secondary caries.

Flexural Strength

Flexural strength was investigated to assess the possible influences of light-curing time and light-curing intensity on the mechanical properties of the tested materials but also to investigate if the influence of these parameters was similar on both mechanical and biological performances. According to ISO 4049/2009[47] specifications, dental restorative materials should have flexural strength values above 80 MPa.[47] Both the tested materials meet this standard, even if silorane-based composite values were inferior to the tested methacrylate-based composite, as already pointed out by another study.[34] This conclusion can validate results from a recent clinical trial study in which most of the failures of silorane-based composite were due to fracture.[45] However, as shown by Goracci and others,[48] other conventional composites have flexural strength values similar to those of silorane-based composite.

The ISO specifications also require the length of the specimens to be 21 mm. Although this method may prove useful to provide completely polymerized specimens, it may not provide accurate information regarding the influence of light-curing parameters on the flexural strength of specimens because the tip of the light-curing-source overlaps during polymerization. For this reason, the length of the bars (10 mm) differed from the ISO specifications and was specifically chosen (as equal to the diameter of the fiberglass tip of the light-cure unit) to allow a single-shot polymerization of the specimens.

Results showed that composite type was the only significant factor ($p<0.0318$) and that light-curing time and light-curing intensity did not influence flexural strength. Consequently, the second null hypothesis could also be rejected.

No threshold value indicating a decrease in mechanical properties was identified for any of the tested light-curing times or light-curing intensities. Even if the manufacturer suggested a polymerization time of 20 seconds, testing after 24 hours from the light-curing process showed that maximum flexural strength values were already reached at 10 seconds, independent of the light-curing intensity tested.

CONCLUSIONS

Within the limits of this study, it is possible to conclude that silorane-based composite is less prone to *S. mutans* biofilm development compared with a widely used methacrylate-based composite. Moreover, silorane surface colonization does not seem to be influenced by such factors as light-curing time and light-curing intensity. This may potentially reduce the occurrence of secondary caries, thus improving the longevity of direct composite restorations. Flexural strength was not influenced by light-curing time or light-curing intensity but proved to be significantly higher for the methacrylate-based composite. It is interesting to note the different influence of light-curing parameters on composite mechanical and biological performances.

Regulatory Statement

This study was conducted at the University of Milan.

Conflict of Interest

The authors of this manuscript certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

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ERRATUM

Operative Dentistry would like to clarify author order in "Brambilla E, Ionescu A, Cazzaniga G, & Ottobelli M (2016) Influence of light-curing parameters on biofilm development and flexural strength of a silorane-based composite *Operative Dentistry* **41(2)** 219-227. The correct author order should be "Ottobelli M, Ionescu A, Cazzaniga G, & Brambilla E." *Operative Dentistry* apologizes for any confusion.