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In vitro biofilm formation on resin-based composites cured under different surface conditions

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ABSTRACT

Objectives: The interfacial conditions occurring during light-curing procedures of resin-based composites (RBCs) influence their surface properties and therefore the biological behavior of the material. This study aimed to evaluate the influence of different surface curing conditions on *in vitro* biofilm formation by *Streptococcus mutans* and mixed oral microflora, in the presence or absence of surface salivary pre-conditioning.

Methods: Two nanohybrid RBCs and four interfacial curing conditions (open air, argon, nitrogen and glycerin) were evaluated. Surface roughness (SR), surface elemental composition (energy-dispersive X-ray spectrometry, EDS) and biofilm formation (*S. mutans* and oral microcosm) were assessed. Surfaces were observed using scanning electron microscopy (SEM). Microbiological tests were performed with and without saliva pre-conditioning of the surfaces. EDS analysis was performed before and after biofilm formation, and biofilm morphology was evaluated using confocal laser scanning microscopy (CLSM). Data were analyzed using multi-way ANOVA and Tukey post-hoc test ($p < 0.05$).

Results: Interfacial curing conditions significantly influenced SR depending on the tested RBC. EDS analysis showed that surface elemental composition was significantly influenced by the interfacial curing condition depending on the tested RBC. Interfacial curing conditions significantly influenced biofilm formation in both microbiological models in the absence of saliva pre-conditioning, depending on the tested RBC, whereas saliva pre-conditioning abrogated these effects.

Conclusions: Surface curing conditions significantly impacted biofilm formation in a material-dependent manner, which was abrogated when surfaces were pre-conditioned with saliva.

Clinical significance: Curing under glycerin did not improve the microbiological performances of the tested RBCs. These results, needing to be confirmed by *in vivo* data, have the potential to simplify operative procedures in restorative dentistry.

1. Introduction

Currently, resin-based composites (RBCs) are the most commonly used restorative materials for both anterior and posterior restorations, because of their ease of use and their high aesthetic performance [1,2]. Nevertheless, secondary caries remains a significant problem for restorations made with these materials [3,4]. An imbalance between the microbial species of the biofilm and the host defenses at the marginal area of a RBC restoration is cited as the main cause of the onset of a recurring lesion [5,6].

Several oral microorganisms are associated with the development of secondary caries, with *Streptococcus mutans* (*S. mutans*) considered one of the main pathogens involved in this disease [7]. The possibility of controlling RBCs' surface properties is therefore seen as a good option to positively influence the relationship between the material and the oral flora, reducing the occurrence of secondary lesions. It has been demonstrated that the surface chemical properties of RBCs are key factors influencing the interactions between the material and oral flora, and the resin curing process plays a crucial role in determining these characteristics [5,8,9]. Experimental data have shown that the surface characteristics and the biological behavior of RBCs are influenced by light-curing parameters, such as the power of the light source and the

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curing time [5,8,10]. The curing process usually occurs in the presence of atmospheric oxygen, which doesn't allow for a complete polymerization of the resin at the surface. This phenomenon leads to the formation of a sticky, uncured oxygen-inhibited layer, thereby requiring a finishing process for removal [11,12]. Furthermore, RBCs can never achieve complete polymerization [13,14], allowing for the presence of monomers and unpolymerized compounds that can be leached out, potentially affecting biofilm formation [10,15–18] This latter effect, however, is still debated [19].

In studies investigating the effects of the oxygen-inhibited layer on RBCs surface features, glycerin insulation has been shown to be the preferred clinical method to protect the RBC surface exposed to the oral environment from oxygen during the curing process [11,20–22]. Other studies have examined the effect of curing composites in inert atmospheres, such as 100% helium, to determine its effect on polymerization shrinkage stress [23]. Nevertheless, there is a lack of data regarding the effect of this curing condition on the surface chemical composition of RBCs and its influence on microbiological behavior.

From a microbiological point of view, the presence of an inert gas during the RBC curing process could modify the surface characteristics of the RBC and thus influence the behavior of the oral bacteria. Argon and nitrogen, two inert gases that are safe for clinical application, may be suitable candidates to investigate the effect of different oxygen-free atmospheres on the curing process. Therefore, the aim of this study was to evaluate the influence of four surface curing conditions (air, argon, nitrogen and glycerin) on *in vitro* biofilm formation by *S. mutans* and by mixed oral microflora, and to determine the effect of saliva pre-conditioning on biofilm formation.

2. Materials and methods

2.1. Specimen preparation

Reagents and disposables used in this study were obtained from Merck (E. Merck AG, Darmstadt, Germany).

Two commercially available nanohybrid RBCs (shade A3, Table 1) differing in monomer content (Bis-GMA and TEGDMA-free RBC 1 vs. conventional BisGMA and TEGDMA-containing RBC 2) and in filler content (fluoro-alumino-silicate glass filled RBC 1 vs. zirconia and silica filled RBC 2) were used to prepare a total of 624 disks by packing each of the two materials into custom-made polytetrafluoroethylene (PTFE) molds placed on the bottom of Petri dishes. The molds contained 24 standardized holes with an internal diameter of 6.0 mm and a thickness of 1.5 mm. The surface of the uncured material was flattened using a soft brush, then the distance between the molds and the lids was adjusted to 1.0 mm. The lids were then hermetically sealed using impression material (Express™ 2 Light Body Standard, 3 M ESPE, Seefeld, Germany), and two holes (diameter = 3 mm) were produced on top of each lid. The holes provided openings for polyethylene tubing that was connected to cylinders containing the tested gases (air, argon, nitrogen) via reduction valves. A constant flow of the gas (1000 ml/min) was maintained inside the Petri dishes for 20 min to obtain the desired atmosphere [24]. The material contained in each well was light-cured through the lids for 60 s, using a quartz-tungsten halogen light (Spectrum 800, Dentsply International Inc., York, PA, USA, with irradiance of 800 mW/cm^2) and with the tip of the unit touching the lid. Given that the thickness of the Petri dish lid was 0.8 mm, the total distance from the light-curing tip to the specimen was 1.8 mm.

Table 1
Codename, type, brand name and composition of the tested RBCs, as specified by the manufacturers.

Codename, Type	Brand Name	Chemical composition	Manufacturer
RBC1 Nanohybrid	Venus Pearl	Filler: Ba-Al-F glass Base resin: TCD-DI-HEA, UDMA	Heraeus Kulzer, Hanau, Germany
RBC2 Nanohybrid	Filtek Supreme XTE	Filler: SiO_2, ZrO_2, aggregated SiO_2-ZrO_2 cluster Base resin: Bis-GMA, Bis-EMA, UDMA, TEGDMA, PEGDMA	3 M, Maplewood, MN, USA

Bis-GMA = bisphenol-A-glycidyl-dimethacrylate; Bis-EMA = ethoxylated bisphenol-A-dimethacrylate; PEGDMA = polyethylene glycol dimethacrylate; TEGDMA = triethylene glycol dimethacrylate; TCD-DI-HEA = 2-propenoic acid, (octahydro-4,7 methano-1H-indene-5-diyl) bis(methyleneiminocarbonyloxy-2,1-ethanediyl) ester ; UDMA = urethane dimethacrylate.

One additional group, cured under a glycerin layer, was included in the experimental design. The curing of these specimens was obtained inside the Petri dishes as previously specified, by covering each specimen with a drop (30 µl) of glycerine, and then curing in absence of air flow at the same distance and conditions as for the other specimens. After that, glycerine was rinsed off from specimens' surfaces using distilled water.

Each Petri dish was then stored under light-proof conditions for 24 h at 37 °C to allow complete polymerization of the RBC. Specimens were then 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. After that, specimens were transferred to sterile Petri dishes containing 25 ml of sterile PBS and stored at room temperature for an additional 7 days to allow unreacted monomers to leach out of the composite disks. During this period, each dish was rinsed with 25 ml of sterile PBS twice a day to remove any leachates.

After that, to assess the surface homogeneity of the samples, surface roughness (SR, n = 5/group) was measured using a profilometer (Sutronic 3+; Taylor Hobson, Leicester, UK). Randomly chosen segments of 1.75 mm were measured in three line scans for each specimen, using a diamond tip with a radius of 2 µm and a tip angle of 90°. The cut-off level was set to 0.25. Data were expressed as Ra (µm).

2.2. Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS) analysis

SEM and EDS analysis were performed on test specimens (n = 3/group) using a TM3030Plus Tabletop scanning electron microscope (Hitachi, Schaumburg, IL, USA) and an EDS probe (SwiftED3000 Oxford Instruments Analytical Ltd., Abingdon, Oxfordshire, UK). Specimens were mounted on stubs using conductive tape and directly observed in surface-charge reduction mode without sputter-coating, using an accelerating voltage of 15 KV. Three randomly selected fields were acquired for each specimen at 5000X magnification to display the influence of the interfacial curing conditions on the surfaces of the tested RBCs. Specimens were also analyzed using the EDS probe before and after storage in PBS. Three randomly selected 300 × 300 µm fields were analyzed for each specimen in full-frame mode using an acquisition time of 150 s at 15 KV accelerating voltage. The acquired data represent the elemental composition of the ≈1 µm superficial layer.

SEM and EDS analyses were repeated on the same specimens following the microbiological procedures. Specimens were sonicated (Sonifier Model B-15; Branson, Danbury, CT, USA at 40 W energy output for 10 min) and carefully cleaned using a microbrush to remove biofilm remnants. In this way, to obtain information about the material's surface behavior, data obtained directly after light-curing can be compared with data acquired after PBS storage, while comparisons between data obtained after biofilm formation and after PBS storage can provide evidence of the mutual interactions between biofilms and materials' surfaces.

2.3. Saliva collection

To pre-condition the surfaces of the tested specimens, whole saliva