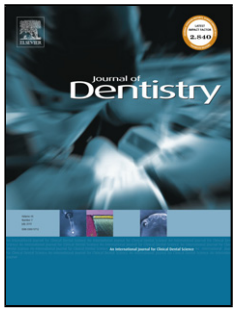

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Authors: Gloria Cazzaniga, Marco Ottobelli, Andrei C. Ionescu, Gaetano Paolone, Enrico Gherlone, Jack L. Ferracane, Eugenio Brambilla

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In vitro biofilm formation on resin-based composites after different finishing and polishing procedures

Short title: Biofilm formation and RBC finishing.

Gloria Cazzaniga (DDS)[1], Marco Ottobelli (DDS)[1], Andrei C. Ionescu (DDS, PhD)[1], Gaetano Paolone (DDS)[2], Enrico Gherlone (MD, DDS)[2], Jack L. Ferracane (DDS, MSc, PhD)[3], Eugenio Brambilla (DDS)[1].

1: Department of Biomedical, Surgical and Dental Sciences, IRCCS Galeazzi Orthopedic Institute, University of Milan, Milan, Italy.

2: Dental Clinic – Restorative Dentistry, Vita-Salute San Raffaele University, Milan, Italy.

3: Department of Restorative Dentistry, Oregon Health & Science University, Portland, OR, USA.

Correspondence:

Prof. Eugenio Brambilla, Department of Microbiology,

Via Pascal 36, First floor, Cap. 20133 Milan, Italy.

E-mail: eugenio.brambilla@unimi.it

Phone: +390250319007

Fax: +390250319040

Abstract

Objectives: To evaluate the influence of surface treatments of different resin-based composites (RBCs) on *S. mutans* biofilm formation.

Methods: 4 RBCs (microhybrid, nanohybrid, nanofilled, bulk-filled) and 6 finishing-polishing (F/P) procedures (open-air light-curing, light-curing against Mylar strip, aluminum oxide discs, one-step rubber point, diamond bur, multi-blade carbide bur) were evaluated. Surface roughness (SR) (n=5/group), gloss (n=5/group), scanning electron microscopy morphological analysis (SEM), energy-dispersive X-ray spectrometry (EDS) (n=3/group), and *S. mutans* biofilm formation (n=16/group) were assessed. EDS analysis was repeated after the biofilm assay. A morphological evaluation of *S. mutans* biofilm was also performed using confocal laser-scanning microscopy (CLSM) (n=2/group). The data were analyzed using Wilcoxon (SR, gloss) and two-way ANOVA with Tukey as post-hoc tests (EDS, biofilm formation).

Results: F/P procedures as well as RBCs significantly influenced SR and gloss. While F/P procedures did not significantly influence *S. mutans* biofilm formation, a significant influence of RBCs on the same parameter was found. Different RBCs showed different surface elemental composition. Both F/P procedures and *S. mutans* biofilm formation significantly modified this parameter.

Conclusions: The tested F/P procedures significantly influenced RBCs surface properties but did not significantly affect *S. mutans* biofilm formation. The significant influence of the different RBCs tested on *S. mutans* biofilm formation suggests that material characteristics and composition play a greater role than SR.

Clinical significance: F/P procedures of RBCs may unexpectedly play a minor role compared to that of the restoration material itself in bacterial colonization.

Keywords: resin composites; finishing; polishing; bioreactor; *Streptococcus mutans*; biofilm.

1. Introduction

Resin-based composites (RBCs) have been widely used for both anterior and posterior restorations because of the increasing demand for high-quality aesthetic results in everyday practice. However, despite the improvements in the performances of these materials, the most frequent reason for replacement is still the development of secondary caries, which influences the longevity of the restorations [1,2]. The biological interactions between restorative materials and the overlying biofilm are a key factor in this process.

Several plaque-associated microorganisms have been related to dental caries, and *Streptococcus mutans* (*S. mutans*) is considered one of the main pathogens involved in the development of this disease [3,4]. Microbial colonization of oral hard surfaces begins at locations, such as surface irregularities, where bacteria can grow protected against hydrodynamic shear forces [5,6]. Furthermore, *in vitro* and *in vivo* studies highlighted that the interactions of RBCs with oral microorganisms are significantly influenced by the surface properties of the material [7]. Among these properties, surface roughness (SR) and surface chemical composition play a crucial role in bacterial adhesion and biofilm formation [9,10]. For this reason, the modulation of RBCs surface properties is of increasing importance from a microbiological point of view.

In recent years, restorative materials have rapidly evolved both in terms of filler particles and resin matrix composition and structure. The application of nanotechnology in the dental materials field has resulted in the development of new RBCs containing nanometer sized particles, called nanofilled composites. Furthermore, a new RBC category, called bulk-filled composites, has recently been introduced. The main characteristic of these materials is to be self-adapting and to offer the opportunity to be used in thick layers, without an increase in the polymerization shrinkage stress or a reduction of the degree of conversion [10,11]. Nevertheless, it is well known that no RBC is able to achieve full conversion, and this negatively influences its microbiological behavior [12].

After an RBC restoration placement, a finishing and polishing (F/P) procedure is used to decrease SR, thereby obtaining a smoother and glossy surface [13]. This procedure is also useful to refine surface anatomy and remove the oxygen-inhibited layer that promotes bacterial adhesion and colonization. For these purposes, a great variety of F/P instruments and techniques are available. Several studies have explored the relationships between F/P procedures and SR [14,15], as well as biofilm formation on RBCs with different surface properties [16,17]. *In vitro* studies are frequently performed on this topic using continuous culture systems under standardized experimental conditions. These devices allow the development of monospecies or multispecies biofilms in conditions close to that of the oral environment [18-20]. Nevertheless, very few studies have investigated the influence of different surface treatment protocols on both surface characteristics and biofilm formation of different commercial RBCs used for direct restorations.

The aim of this study was therefore to evaluate the effect of 6 different F/P protocols on SR, gloss, chemical

composition and *S. mutans* biofilm formation on the surfaces of 4 commercially available RBCs using a continuous-flow bioreactor model.

2. Materials and Methods

2.1. Specimen Preparation

Four commercially available RBCs, with different types of filler particles, were used in the present study, as described in Table 1. Ten syringes of each RBC (shade A3) were used to prepare a total of 108 discs by packing an excess of uncured composite into a custom-made polytetrafluoroethylene (PTFE) mold with a diameter of 6.0 mm and a thickness of 1.5 mm. After that, the material was covered with a Mylar strip on the top and bottom surfaces of the PTFE mold and condensed against a glass plate. The specimens were then irradiated for 80 s by placing the tip of a hand-held light-curing unit (Spectrum 800; Dentsply International Inc., York, PA, USA) into direct contact with the Mylar strip. The specimens were then randomly divided into 6 groups (Group 1 - Group 6). Specimens belonging to Group 1 (n=18) were prepared as described above but they were light-cured for 80 s by placing the tip of the light-curing unit at 1 mm distance from the surface after removing the Mylar strips, to simulate open-air polymerization.

The following F/P procedures were tested for each RBC on the remaining specimens ~~randomly divided into~~:

- Group 2 (n=18) no finishing (Mylar strip)
- Group 3 (n=18) Al_2O_3 discs (Sof-Lex, 3M, Maplewood, Minnesota, USA)
- Group 4 (n=18) one-step rubber points (Opti1Step, Kerr Corp., Orange, California, USA)
- Group 5 (n=18) diamond bur 8862.314.012 followed by 862EF.314.012 (Komet, Gebr. Brasseler GmbH & Co. KG, Lemgo, Germany)
- Group 6 (n=18) multi-blade carbide bur H48L.Q.314.012 followed by H48L.UF.314.012 (Komet, Gebr. Brasseler GmbH & Co. KG, Lemgo, Germany).

Group 2 specimens were not furtherly processed. Group 3 specimens were finished and polished with a sequence of three Al_2O_3 discs (medium, fine, superfine) for 30 s each at 20,000 rpm under water irrigation. After each step the specimens were washed with distilled water and air dried for 10 s. After every three specimens, discs were changed with new ones to obtain surfaces with homogeneous characteristics. Group 4, 5 and 6 specimens were treated for 30 s at 20,000 rpm under water irrigation and a new point/bur was used every three specimens.

After that, all specimens were ~~carefully removed from the mold,~~ placed into the wells of a 48-well plate (Nunc, Roskilde, Denmark) and stored under light-proof conditions in distilled water for 6 days at 37 ± 1°C. To reduce the amount of residual monomer that may leak during the subsequent incubation, each specimen was rinsed twice a day

with 1 ml of distilled water. Specimens ~~undergoing surface analyses~~ were subsequently carefully cleaned using an applicator brush tip (3M, Maplewood, Minnesota, USA) soaked in ethanol (70%) prior to any further processing.

2.2. Roughness measurements

The SR of the specimens (n=5/group) was measured using a profilometer (Sutronic 3+; Taylor Hobson, Leicester, UK). A distance of 1.75 mm was measured in three line scans perpendicular to the expected grinding grooves for each specimen, using a standard diamond tip (tip radius 2 µm, tip angle 90°) and a cut-off level of 0.25. Data were expressed as Ra.

2.3. Gloss measurements

Gloss measurements (n=5/group), expressed as gloss units (GU), were performed using a small-area glossmeter (MG6-SA; KSJ, Quanzhou, China) with a square measurement area of 2 x 2 mm and a 60° geometry. A black opaque plastic mold was placed over the specimen during measurements to avoid the influence of the ambient light and to maintain the exact position of the specimen. Three measurements were performed for each specimen.

2.4. SEM and 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) equipped with an EDS probe (SwiftED3000 Oxford Instruments Analytical Ltd., Abingdon, Oxfordshire, UK). Three randomly selected fields were acquired for each specimen at 5000x magnification to display the influence of the F/P procedures on the surfaces of the tested RBCs. For the EDS analysis, three randomly selected 300 x 300 µm fields were analyzed for each specimen in full-frame mode using an acquisition time of 150 s at 5 and 15 KV accelerating voltage. Acquired data represent the elemental composition of 1 µm superficial layer from which electrons were extracted by the accelerated beam. SEM and EDS analyses were repeated on the same specimens following the microbiological procedures described in paragraph 2.6. Specimens were sonicated (Sonifier Model B-15; Branson, Danbury, CT, USA operating at 40 W energy output for 10 min) and carefully cleaned using a microbrush to remove any biofilm.

2.5. Saliva collection

Paraffin-stimulated whole saliva was collected from three healthy volunteer donors in accordance with the protocol published by Guggenheim *et al.* [21]. Briefly, saliva was collected in chilled test tubes, pooled, heated to 60°C for 30 min to inactivate endogenous enzymes and was then centrifuged (12,000 rpm for 15 min at 4°C). The supernatant was transferred into sterile tubes, stored at -20°C, and thawed at 37°C for 1 h prior to the experiments.

2.6. Microbiological procedures

Culture media were obtained from Becton-Dickinson (BD Diagnostics-Difco, Franklin Lakes, NJ, U.SA) and reagents

were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, U.S.A.). Mitis Salivarius Bacitracin agar (MSB agar) plates were inoculated with *S. mutans* (ATCC 35668) and incubated at 37°C for 48 h in a 5% CO_2-supplemented environment. A pure culture of the microorganism in Brain Heart Infusion broth (BHI) was obtained from these plates after incubation at 37°C for 12 h in a 5% CO_2-supplemented environment. Cells were harvested by centrifugation (2,200 rpm, 19°C, 5 min), washed twice with sterile phosphate-buffered saline (PBS) and resuspended in the same buffer. The cell suspension was subsequently subjected to sonication (Sonifier model B-150; Branson, Danbury, CT, USA; operating at 7W energy output for 30 s) to disperse bacterial chains. Finally, the suspension was adjusted to a McFarland scale 1.0 optical density, corresponding to a concentration of approximately 6.0 x 10^8 cells/mL.

2.7. MDFR procedures

The drip flow reactor (MDFR) used in the study was a modification of a commercially available Drip Flow Reactor (DFR 110; BioSurface Technologies, Bozeman, MT, USA). The modified design allowed the placement of customized specimen trays on the bottom of the flow cells and the complete immersion of the RBC surfaces into the surrounding flowing medium [17,18]. All specimens (n=18/group) were placed in a PTFE tray on the bottom of the flow cell and exposed to the surrounding medium. All tubing, specimens, and the specimen-containing trays were sterilized before incubation using a chemiclave (Sterrad; ASP, Irvine, CA, USA). By limiting the maximum temperature to 45°C, heat-related damage of the RBC specimens was avoided. The whole MDFR was then assembled inside a sterile hood and transferred into an incubator at 37°C.

A total of 10 ml of thawed sterile saliva was placed into each flow cell and the MDFR was then incubated at 37 °C for 24 h to allow the formation of a salivary pellicle on the surface of the specimens. After this incubation, saliva was removed by gentle aspiration and each flow cell was inoculated with 10 ml of *S. mutans* suspension to allow bacterial colonization of the RBCs surfaces. After 4 h, a multichannel, computer-controlled peristaltic pump (RP-1; Rainin, Emeryville, CA, USA) was turned on to provide a constant flow of nutrient medium through the flow cells. Sterile culture medium including 2.5 g/L mucin (type II, porcine gastric), 2.0 g/L bacteriological peptone, 2.0 g/L tryptone, 1.0 g/L yeast extract, 0.35 g/L NaCl, 0.2 g/L KCl, 0.2 g/L CaCl2, 0.1 g/L cysteine hydrochloride, 0.001 g/L hemin, and 0.0002 g/L vitamin K1, supplemented with 1% sucrose was used. The flow rate was set to 9.6 ml/h [18]. Viable biomass assessment (n=16/group) and CLSM microscopy (n=2/group) were performed after 24 h of incubation.

2.8. Viable biomass assay

A tetrazolium salt stock solution was prepared by dissolving 5 mg/mL of 3-(4,5)-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) in sterile PBS. A phenazinium salt stock solution was prepared by dissolving 0.3 mg/mL of N-methylphenazinium methyl sulfate (PMS) 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 (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 sulfate and 50% v/v dimethylformamide in distilled water. It was stored at 2°C until the day of the experiment and warmed at 37°C for 2 h before use.

After a 24 h of incubation, the medium flow was stopped and the flow cells were opened. The trays containing the specimens were placed into Petri plates containing sterile PBS at 37°C. Specimens were gently removed from the trays, washed three times with sterile PBS to remove non-adhered cells and finally placed in 48-well plates. The MTT assay was performed as follows [22]: 300 µl of FMS were placed in each well, then the plates were incubated for 3 h in light-proof conditions at 37°C. Electron transport across the microbial plasmatic membrane and, to a lesser extent, microbial redox systems, converted the yellow MTT salt to insoluble purple formazan during the incubation. The conversion at the microbial membrane level was facilitated by the intermediate electron acceptor (PMS). The FMS was then gently removed by aspiration and the intracellular formazan crystals were dissolved by adding 300 µl of lysing solution to each well. Plates were then incubated for 1 h at room temperature in lightproof conditions. The optical density (550 nm) of 100 µl of the suspension in each well was measured with a spectrophotometer (Genesys 10-S; Thermo Spectronic, Rochester, NY, USA). Results were recorded as OD units and were proportional to the amount of viable and metabolically active cells adherent to the sample surface [22].

2.9. CLSM morphological analysis

Two specimens for each group were analyzed using CLSM. After a 24 h incubation, the biofilm growing on the specimens was gently washed three times with PBS to remove non-adherent cells and stained using the FilmTracer live/dead Biofilm Viability Kit (Invitrogen, Carlsbad, California, USA). Biofilm was observed using a confocal laser scanning microscope (Leica TCS SP2; Leica Microsystems, Wetzlar, Germany). Three randomly selected image stack sections were recorded for each specimen. Confocal images were obtained using a dry 20× (NA 0.7) objective and digitalized using Leica Application Suite Advanced Fluorescence (LAS AF; Leica microsystems, Wetzlar, Germany), at a resolution of 1024 × 1024 pixels, with a zoom factor of 1.0. For each image stack section, 3D-rendering reconstructions were obtained using Drishti 3D software [23].

2.10. Statistical analysis

Data were analyzed using statistical software (JMP 12 Pro; SAS, Cary, NC, USA) with a significance level of 5%. The variables gloss and viable biomass showed normal (Shapiro-Wilk's test) and homogeneous (Levene test) distributions. The two variables were submitted to 2-way ANOVA considering RBC and F/P procedures as fixed factors, followed by the Tukey test. SR data were log-transformed to approach a normal distribution, then were submitted to 2-way ANOVA, followed by the Tukey test.

Regression analyses were performed to determine any possible correlation between gloss and SR parameters.

3. Results

3.1. Surface roughness

Mean Ra values ± 1 standard error are shown in **Fig. 1a**. A significant influence of the tested RBCs on SR was found. HFO (microhybrid) showed lower SR values compared with the other RBCs tested ($p<0.001$). No statistically significant differences were found in SR results among Estelite Asteria (nanohybrid), Filtek Supreme XTE (nanofilled) and Sonicfill 2 (bulk-filled) RBCs.

A significant influence of the tested F/P procedures on SR was also observed. Group 2 (Mylar) showed the lowest SR values ($p<0.001$). Group 3 (Al_2O_3 discs) showed lower SR values than Groups 4 (one-step rubber points), 5 (diamond bur) and 6 (multi-blade carbide bur) ($p<0.001$). No statistically significant differences were found in SR results among Groups 4, 5 and 6. Group 1 (no finishing) showed the highest SR values.

A significant interaction between the two factors (F/P procedures and RBC) was found ($p<0.001$).

3.2. Gloss

Mean GU values ± 1 standard error are shown in **Fig. 1b**. A significant influence of the tested RBCs on SR was observed. HFO (microhybrid) and Estelite Asteria (nanohybrid) RBCs showed the highest gloss values, while Sonicfill 2 (bulk-filled) RBCs showed the lowest ($p<0.001$). Filtek Supreme XTE (nanofilled) RBCs showed intermediate gloss values.

A significant effect of F/P procedures ($p<0.001$) on surface gloss was demonstrated. Group 2 (Mylar) showed the highest gloss values, while Groups 1 (no finishing) and 5 (diamond bur) yielded the lowest. Groups 3 (Al_2O_3 discs), 4 (one-step rubber points) and 6 (multi-blade carbide bur) showed intermediate gloss values.

A significant interaction between the two factors (F/P procedures and RBC) was found ($p<0.001$).

Fig. 1c shows the correlation between SR and gloss. For all RBCs a significant linear negative correlation was found between the two parameters ($p<0.01$). The correlation was relatively strong for HFO (microhybrid) whereas for the other RBCs it was weaker.

3.3. SEM and EDS

Differences in surface texture for each group, prior to biofilm formation, are shown in **Fig. 2**. All the RBCs have a heterogeneous surface displaying inorganic fillers and organic resin matrix. For all materials Groups 1 and 2 (no finishing and Mylar, respectively) showed filler particles surrounded by a coating layer of resin matrix. Fillers of Filtek Supreme XTE could not be observed at this magnification. All other groups showed superficial grooves left by the F/P procedures. The grooves were barely noticeable in Group 3 (Al_2O_3 discs), which overall appeared to be the F/P

procedure to leave the smoothest surface, confirming SR data. The most evident differences were shown for Sonicfill 2 (bulk-filled) RBCs, with surface exposed macro-sized filler particles.

EDS analysis, performed prior to biofilm formation, showed marked differences in the elemental composition of the tested RBCs (**Table 2**). To compare the influence of the different RBCs and F/P procedures on surface composition, all the elements belonging to the inorganic fraction were summed, whereas the organic fraction was represented by carbon. Oxygen was detected but not considered in the analysis since it was representative of both the organic (polymers) and the inorganic (oxides) fractions. No evidence of foreign elements on the RBC surfaces after the F/P procedures due to contamination from the finishing discs, rubbers or burs was observed. A significant influence of the tested RBCs and F/P procedures on organic and inorganic fractions was found both before and after biofilm formation ($p<0.001$), as well as a significant interaction between the two factors ($p<0.001$).

Fig. 3 shows the pattern of organic and inorganic fractions detected by EDS as representative of the resin matrix and fillers before and after biofilm formation.

Considering data acquired before biofilm formation (**Fig. 3a**), Group 1 specimens presented a higher amount of organic matrix than inorganic filler in all the tested RBCs except for Filtek Supreme XTE (nanofilled). The latter showed the highest proportion of inorganic filler on the surface, independent of F/P procedure. In Group 2, Enamel plus HFO (microhybrid) and Estelite Asteria (nanohybrid) RBCs displayed a similar proportion of organic and inorganic fractions. Sonicfill 2 (bulk-filled) RBCs showed a higher organic fraction, while the opposite was found for Filtek Supreme XTE RBCs. Groups 3 to 6 presented a similar pattern, showing a higher amount of inorganic filler in all the tested RBCs except for Estelite Asteria, in which inorganic and organic fractions were present in comparable amounts.

After biofilm formation (**Fig. 3b**) an increase in carbon content was observed in all experimental groups.

Fig. 4 shows biofilm-related structures adhering to the RBC surfaces after biofilm formation and cleaning procedures. All specimens observed by SEM and EDS after biofilm formation showed similar features: they appeared to be dotted with dark gray islets of homogeneous material without any evidence of bacterial cells. The acquired elemental maps (**Fig. 4c** and **4d**) showed that the islets were composed of carbon and traces of other elements (mostly N, as displayed, but also Na, Ca, and P), suggesting their biological origin.

3.4. Biofilm formation

Biofilm formation on RBC discs is shown in **Fig. 5.** Two-way ANOVA demonstrated a significant influence of RBC ($p = 0.007$) on biofilm formation. Filtek Supreme XTE RBCs showed significantly lower biofilm formation when compared to Sonicfill 2 RBCs. No significant differences were found between the other RBCs analyzed. Considering the different F/P procedures tested, no significant effect ($p = 0.310$) on biofilm formation could be found.

A significant interaction between the two factors (F/P procedures and RBC) was found ($p=0.047$).

CLSM observations did not show morphological differences in biofilm formation or in its viability among the different F/P groups or RBC materials. All fields showed mature, compact biofilm structures covering most part of the specimen surfaces with a prevalence of viable cells.

4. Discussion

A variety of F/P procedures is used in clinical practice to obtain excellent aesthetic performances of composite restorations [24,25]. Nevertheless, there is still no clear evidence of the influence of these procedures on the biological interactions of RBCs with oral biofilms. ~~This study evaluated four commercially available RBCs treated with six standardized F/P procedures to assess their influence on *in vitro S. mutans* biofilm formation.~~ To provide a broad perspective of the clinical situations, the RBCs tested in the present study belong to four classes of RBCs currently used in daily practice and include a microhybrid, a nanohybrid, a nanofilled and a bulk-filled composite. The influence of the material on biofilm formation is due to different factors such as filler particle characteristics and matrix chemical composition. It has been long known that biofilm formation on RBC surfaces is higher than on those of other restorative materials and sound enamel [26-28]. These data support the hypothesis that RBC susceptibility to bacterial colonization could be due to a specific microbial affinity with the surfaces of these materials. Mechanisms involved in bacterial adhesion and biofilm formation are based on initial physico-chemical interactions between microbial cells and the surfaces of the materials. These interactions are followed by a specific process involving bacterial adhesins and specific sites on the salivary pellicle covering the material [29-31]. ~~Literature data indeed show that the adhesion process of a bacterial strain on the material that acts as an adhesion substrate is highly specific [31].~~ Furthermore, RBC polymerization is never complete, which leads to a leakage of both unpolymerized monomers and biodegradation products from the material surface. It was therefore hypothesized that released monomers may stimulate the growth of bacteria, promoting biofilm formation. This assumption, however, is still controversial [12,32,33].

Each material was submitted to surface treatments that simulated clinical procedures. Among the tested F/P procedures, a “no surface treatment” group (Group 1) was introduced to simulate restoration areas difficult to reach and therefore often not involved in F/P procedures. This group was characterized by the presence of an oxygen-inhibited layer. Group 2 included specimens cured against Mylar strips that are frequently used to build proximal areas, especially in anterior teeth. This group allowed an RBC surface cured in an oxygen-free environment and a very low SR to be tested. Aluminum oxide discs (Group 3) usually represent a standard protocol to obtain smooth surfaces on a variety of RBCs [34]; the one-step polishing system (Group 4) was chosen as a fast way to obtain a smooth surface with a single instrument [14,35]. Diamond burs (Group 5) are used to shape the anatomy of restorations thanks to their high cutting efficiency, while multi-blade carbide burs (Group 6), showing low cutting efficiency, are best suited for smoothing and

finishing [36]. ~~In this study specimens were submitted to a manual preparation performed by a single expert operator to better simulate clinical conditions and remove the possible bias related to variations in operators' experience and skills.~~

The study was performed using *S. mutans* as test microorganism since it is directly involved in the etiology of dental caries [3,4,37]. The evaluation of *S. mutans* interactions with the tested surfaces could therefore be of great interest in preventing cariogenic biofilm formation and increasing restoration longevity.

Considering the problems related to the design of *in vivo* studies under controlled cariogenic conditions, the use of a continuous-flow bioreactor represents a valuable possibility, allowing the study of biofilms under dynamic conditions closely simulating *in vivo* ones [38]. In addition, the use of an MDFR allowed us to select an incubation time suitable for the study of the biofilm formation phase. Several studies available in the literature, in fact, evaluated adhesion and the first steps of biofilm formation on RBCs considering the influence of surface properties [6,7,17-20,24,30,31,39-48], while in the present study, the formation of a multilayer biofilm was simulated using a 24 h incubation time to assess the influence of the tested parameters on a structured, multilayered biofilm.

Another important factor influencing biofilm formation on RBC surfaces is the presence of a salivary pellicle. This structure can alter the nanotopography of surfaces [49], thereby significantly modifying the effect of main surface parameters such as roughness or chemical composition. The influence on the latter parameter is not completely clear. Experimental data suggest that the original material surface properties could be transferred even through the pellicle protein layer (shine-through effect) and still influence microbial adhesion [50,51]. However, it is also likely that the influence of those properties decreases over time as biofilm formation takes place and bacteria react to one another as opposed to a fresh surface [52]. Park and coworkers in 2012 investigated the influence of SR on biofilm formation, testing the surface of a nanofilled RBC [46]. The authors concluded that the effect of SR on biofilm formation was not significantly influenced by saliva conditioning. On the contrary, Pereira and coworkers in 2011 demonstrated that saliva conditioning promotes a significant increase in biofilm formation on three RBC surfaces regardless of the F/P procedures applied [45]. In this study, it was therefore decided to condition the surfaces of the tested RBCs with saliva prior to biofilm formation.

SR is one of the most studied surface properties in dental biofilm research. It is also considered to be very important from clinical point of view because of its influence on the aesthetic performances of a restorative material. In agreement with literature data, our results indicate that SR is significantly influenced both by the material and by the F/P procedures [13-15,25,34,53,54]. From the materials point of view, it is generally believed that the result of finishing and polishing RBC surfaces is influenced by filler particle size. Literature data show that F/P procedures applied to RBCs with smaller sized filler particles result in smoother surfaces than on RBCs with larger sized filler particles [14,53,54]. The results of our study do not allow us to confirm these statements since the RBCs tested in this study have a complex

filler composition, with the concurrent presence of particles differing in structure and size. This factor makes it difficult to effectively relate SR data to RBC filler composition [15,44,55]. From the F/P procedures point of view, the smoothest RBC surfaces were obtained by curing specimens against Mylar strips (Group 2), as already shown in the literature [14,18,25]. Group 3 (Al_2O_3 discs), showed intermediate SR values, while Groups 4 (one-step rubber point), 5 (diamond bur) and 6 (multi-blade carbide bur) showed higher SR. The results of the Al_2O_3 discs were probably due to their protocol, which includes treatment with four different discs with progressively finer grit size to abrade both resin matrix and filler, leaving smooth surfaces with almost no grooves (**Fig. 2)**.

Gloss is commonly used to measure the surface shine and therefore influence the aesthetic appearance of composite restorations [14,15]. The one-step system (Group 4) showed the highest gloss values among the F/P procedures except for specimens cured against Mylar strips (Group 2). Several studies demonstrated the existence of a strong correlation between SR and gloss [15,42]. Our evaluations confirm the existence of a significant inverse linear correlation between these two factors for all the tested RBCs. This correlation was shown to be material-dependent since it was stronger for the microhybrid composite (Enamel Plus HFO) and weaker for the other RBCs (**Fig. 1**).

EDS analysis was performed to clarify the relationships between the elemental composition of the tested surfaces and biofilm formation. ANOVA results showed that F/P procedures significantly affected surface chemistry depending on the tested RBC both before and after biofilm formation. After the cleaning procedures of the specimens' surfaces, no differences in the organic fraction were expected. Surprisingly, biofilm residues (likely exopolysaccharide matrix) were still present on all the specimens' surfaces and an increase in this parameter was observed in all experimental groups. Therefore, the organic fraction found after biofilm formation represents the sum of carbon derived from resin matrix and biofilm residues (**Fig. 4**). Nevertheless, as confirmed by literature data, no simple and immediate relationship has been found between the surface elemental composition of RBCs and biofilm formation [7]. For instance, in this study a reduced biofilm formation was expected on the surface of Filtek Supreme XTE RBC containing fluoride filler particles (Ytterbium fluoride, YbF^3, as claimed by the manufacturer and confirmed by EDS analysis). Biofilm data did not support these findings, and it may be speculated that the amount of fluoride in the material composition or its release were too low to show any significant reduction in biofilm formation. These observations were confirmed by the EDS analysis performed after biofilm formation, which showed similar amounts of YbF^3 as prior to biofilm formation.

Microbiological data showed that *S. mutans* biofilm formation was significantly influenced by the material but not by the F/P procedures. From the materials point of view, the nanofilled RBC showed a significantly lower biofilm formation when compared with the bulk-filled RBC. These results agree with those of Steinberg *et al.* who, evaluating *S. sobrinus* adhesion, demonstrated the existence of differences in adhesion of this microorganism to the three microhybrid RBCs tested [39]. Ikeda *et al.*, using an artificial mouth system, found a significant influence of material

on biofilm formation while testing two RBCs for indirect restoration [6]. Pereira *et al*. demonstrated a lower biofilm formation on a nanofilled RBC than on nanohybrid and microhybrid RBCs [45]. These experimental findings suggest that biofilm formation is influenced by the characteristics of the material, including filler size, shape and distribution as well as matrix composition.

From the F/P procedures point of view, it must be highlighted that in the literature these procedures are considered to be able to influence biofilm formation, affecting both SR and other factors related to the surface characteristics. A decrease in SR lowers bacterial adhesion by reducing the contact area between surface and microbial cells [56]. An increase in SR enhances biofilm formation by reducing the shear force on communities growing under flow conditions [9,24,31, 18]. An Ra of 0.2 μm is generally accepted to be the cut-off value below which no correlation between SR and biofilm formation can be found [8]. However, the morphological features of bacterial cells as well as several environmental factors can influence the effects of SR on bacterial adhesion and biofilm formation processes [24]. In our study, no correlation between *S. mutans* biofilm formation and the F/P procedures tested was demonstrated. The main parameter influenced by the F/P procedures, SR, also did not significantly correlate with biofilm formation. It could be hypothesized that the irregularities due to SR could protect bacteria against shear forces during the early stages of biofilm formation, while this parameter seems less important in influencing a fully-grown biofilm [52]. Since F/P procedures also change surface properties such as topography [46] and elemental surface composition [57], as demonstrated in this study, it can be argued that these properties might play an important role in *S. mutans* biofilm formation even exceeding that of SR, especially when considering mature *S. mutans* biofilms.

Our results are in agreement with those of Yamamoto *et al.*, who observed no relationship between SR values and *S. oralis* adhesion to a supra-nano RBC [40]. Eyck *et al*. in 2004 studied *S. mutans* biofilm formation on the surface of restorative materials including a microhybrid RBC using a continuous flow system [41]. Their results showed no correlation of SR with the number of adherent *S. mutans* cells. Three years later, Ono *et al*. demonstrated that different F/P procedures do not influence biofilm formation on the surfaces of a microhybrid or nanohybrid RBC [20]. Aykent *et al.* failed to demonstrate a significant correlation between SR and *S. mutans* biofilm formation under static conditions [42]. Pereira *et al*., comparing a microhybrid, a nanohybrid and a nanofilled RBC submitted to different F/P procedures, found the lowest biofilm formation in the nanofilled RBC but no influence of F/P procedures, in agreement with our findings [45]. In a previous study by our group [18], using the same microbiological model, less *S. mutans* biofilm formation was observed on composite surfaces polished with aluminum oxide discs than on specimens cured against Mylar strips. It was therefore supposed that the higher amount of inorganic components on polished specimens might be responsible of their better biological performances. Nevertheless, the experimental set-up of that study evaluated *S. mutans* biofilm formation after 48h and 96h incubation without the presence of a salivary pellicle [18]. In the present

study specimens were conditioned with human sterile saliva for 24 h to allow the formation of a salivary pellicle. Thus, differences in the experimental set-up of these two studies can explain the differences in the findings, despite the fact that two F/P groups and one RBC were directly comparable.

On the other hand, some authors demonstrated an influence of SR on microbial adhesion. Ikeda *et al.* in 2007 found a significant influence of F/P procedures and SR on biofilm formation, testing two RBCs for indirect restoration [6]. Four years later Mei *et al.*, studying streptococcal adhesion forces to the surface of two orthodontic RBCs with different surface roughness, concluded that this parameter was positively related to SR, highlighting interspecific differences [43]. Yuan *et al.* very recently demonstrated that early adhesion of *S. mutans* to nanofilled and nanohybrid RBCs surface was significantly affected by SR [48].

The results of the present work should be interpreted within the limitations of an *in vitro* study. The main limitation of our *in vitro* approach is the use of a monospecies *S. mutans* biofilm. Considering its role in caries etiology, it nevertheless represents a useful model of a pathogenic biofilm. Further *in vitro* studies are therefore needed to clarify the influence of the tested parameters on the development of a multispecies biofilm in controlled conditions. These preliminary evaluations should be followed by *in situ* and *in vivo* studies to confirm *in vitro* results.

5. Conclusions

The results of this study showed that a clear and immediate relationship between surface characteristics, such as gloss and roughness, and *S. mutans* biofilm formation could not be established. The influence of the different composite materials on biofilm formation was, however, significant. Material characteristics and chemical composition play an important role in biofilm formation processes. F/P procedures did not influence biofilm formation.

Conflict of interest

No conflict of interest exists for any of the authors of this paper.

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Figure legend

Fig. 1 ~~Results of surface roughness and gloss analysis~~. (a): Surface roughness of the tested RBCs splitted by finishing/polishing group. Mean Ra ± SE values are shown. (b): Gloss of the tested RBCs splitted by finishing/polishing group. ~~finishing/polishing groups and RBCs~~. Mean gloss units (GU) ± SE values are displayed. (c): Correlation between the two parameters for each tested RBC. A significant ($p<0.01$) negative linear relationship was found for each RBC; the best goodness of fit was found for HFO (microhybrid).

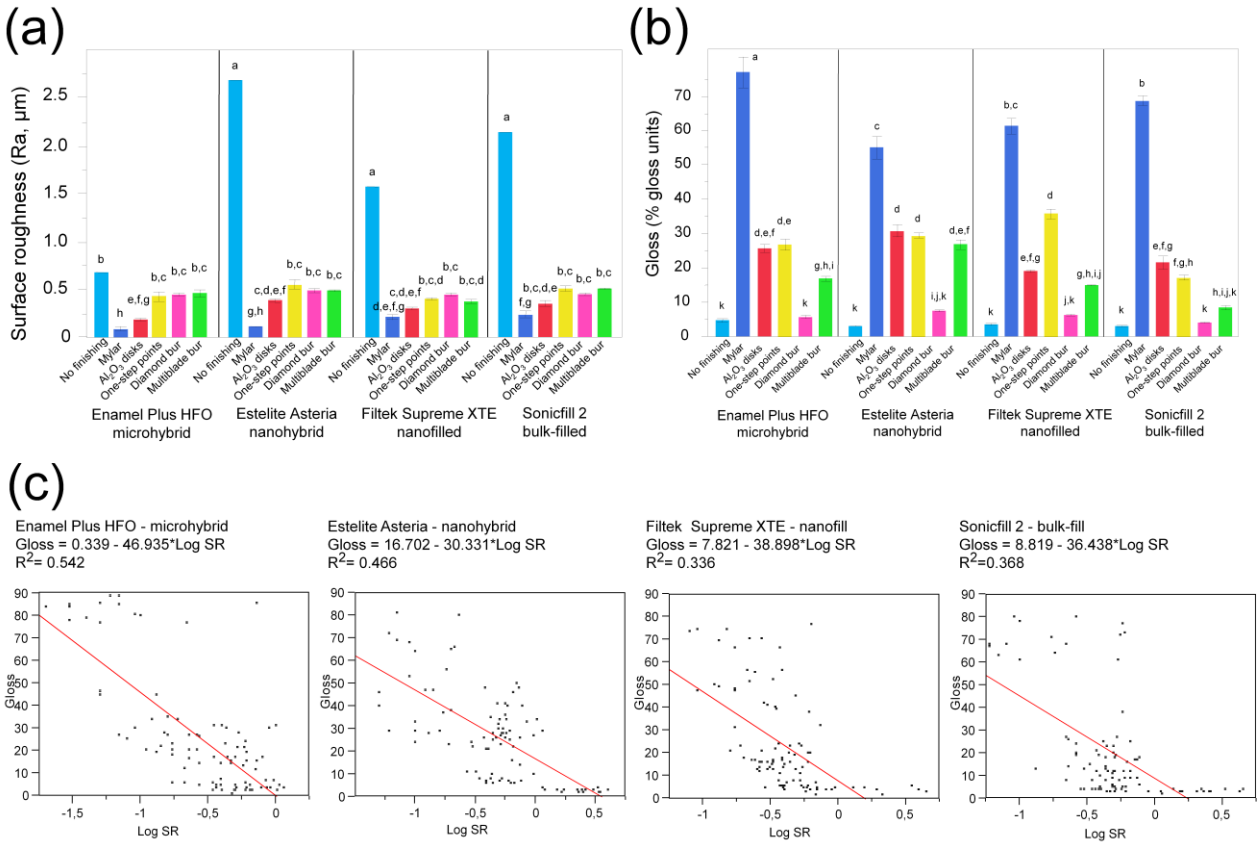


Fig. 2 SEM micrographs of ~~the tested~~ RBC surfaces after ~~application of~~ the tested ~~different~~ F/P procedures (5000x). ~~No differences could be seen between Groups 1 and 2, between Groups 3 and 4 and between Groups 5 and 6, therefore only~~ Micrographs for Groups 2, 3, and 5 are shown. Groups 1 and 2 showed smooth surfaces where filler particles were surrounded by a coating layer of resin matrix. ~~nanofilled material fillers could not be observed at this magnification~~. All other groups showed superficial grooves left by the F/P procedures. ~~The grooves were barely noticeable in Group 3 appeared to be the F/P procedure to leave the smoothest surface except Group 2.~~ F/P procedures in Groups 3 to 6 exposed more filler to the surface compared to Groups 1 and 2. ~~and the bulk-filled RBC surface in particular changed significantly by exposing mini-filler particles.~~

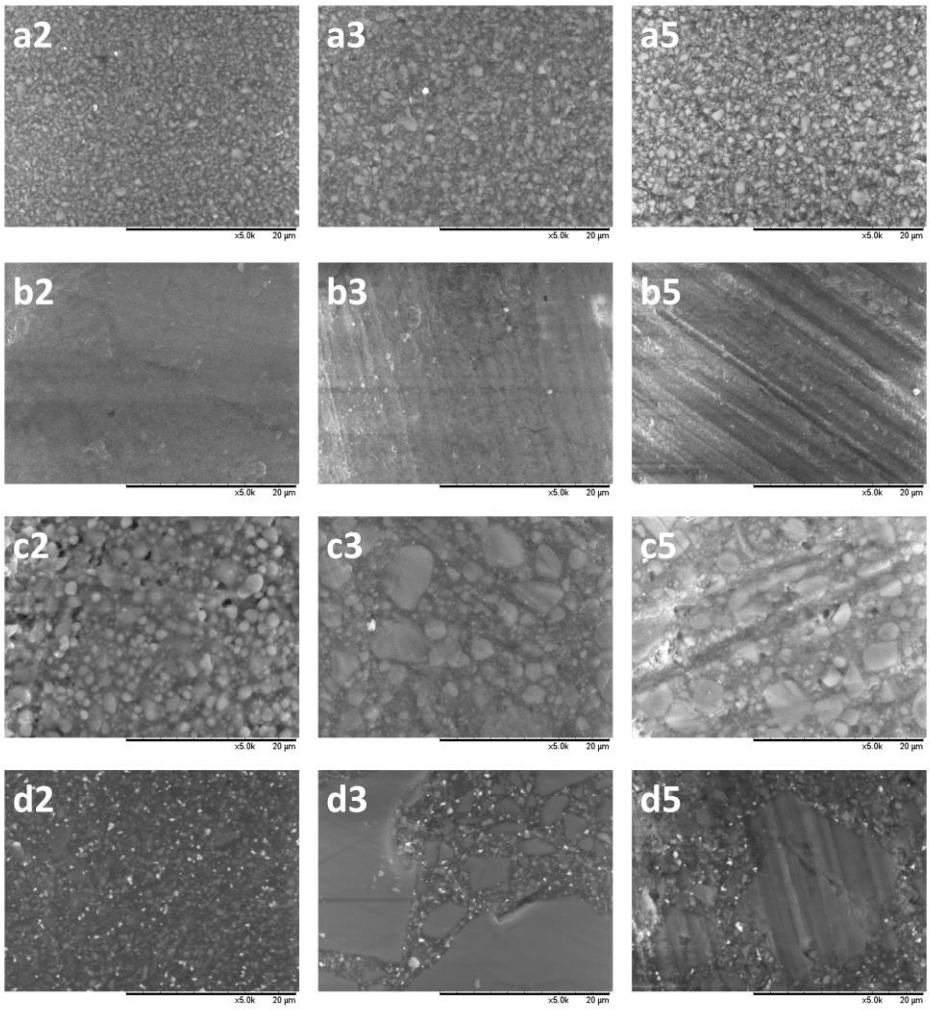


Fig. 3 Surface elemental composition as assessed by EDS analysis. Data are displayed as % of organic fraction (carbon) and inorganic fraction (sum of all elements in RBC fillers). Graph (a) shows data acquired before biofilm formation, while (b) after biofilm formation and cleaning procedures of the specimens ~~(surface rubbing with microbrush and sonication)~~. In this case, organic fraction is a sum of signals from the RBC resin matrix and the residual biofilm (likely exopolysaccharide matrix). Bacterial colonization caused an increase in organic fraction in all groups.

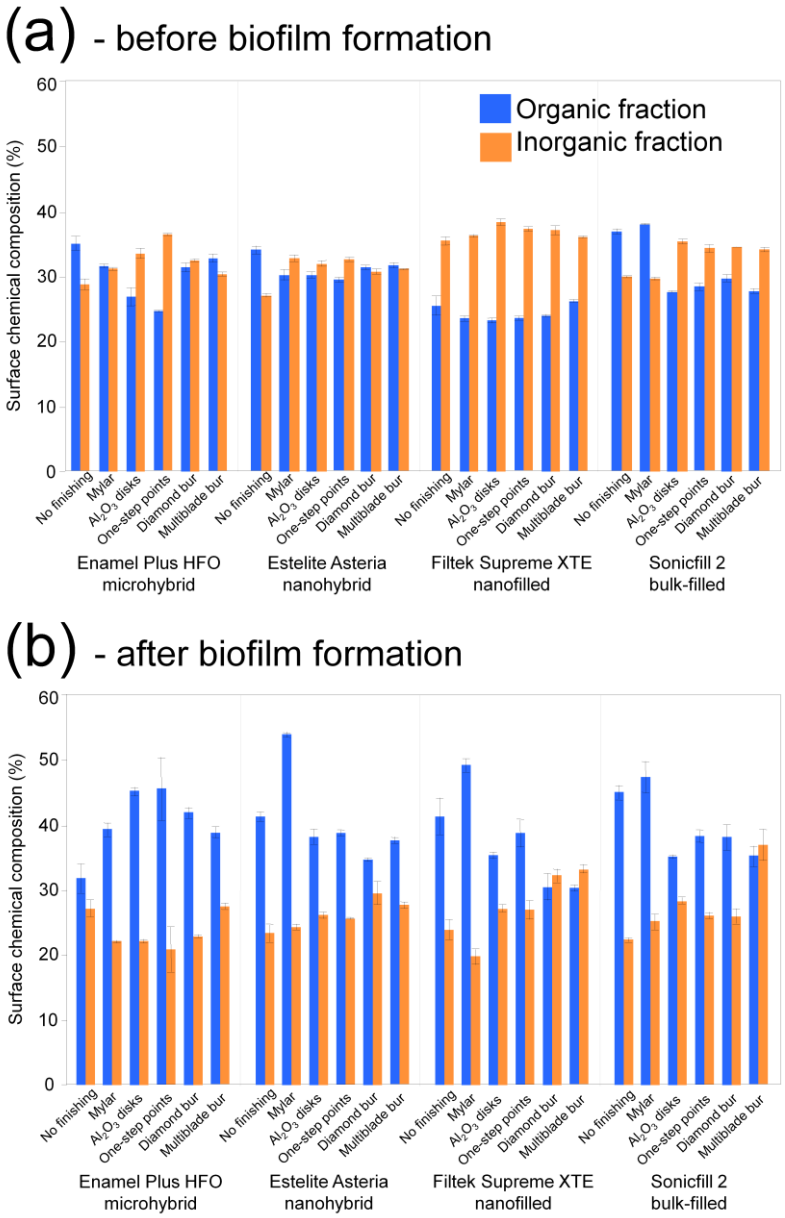


Fig. 4 SEM micrographs of a test specimen surface after biofilm formation ~~Fields acquired for the~~ (Group 3, bulk-filled material). (a) ~~show the surface at 500x magnification and~~ micrograph shows information from the first 0.5 µm of the specimen surface layer while (b) from a greater depth (≈1 µm). ~~All specimens observed after biofilm formation showed similar features: they~~ Specimens appeared to be dotted with dark gray islets of homogeneous adherent material, without recognizable bacterial cells. (c) and (d) show the superimposed EDS elemental map ~~on the SEM images~~. Carbon is displayed in red and silicon ~~channel~~ in blue ~~as markers~~ indicating, respectively, ~~of~~ organic and inorganic fractions. ~~The~~ ~~n~~Nitrogen signal (green) demonstrates that the islets are made of organic material not belonging to the RBCs. ~~The maps indeed show that the islets were composed by carbon and traces of other elements (N, Na, Ca, P), suggesting that,~~ ~~m~~Most likely islets are biofilm residues still firmly attached to the surface.

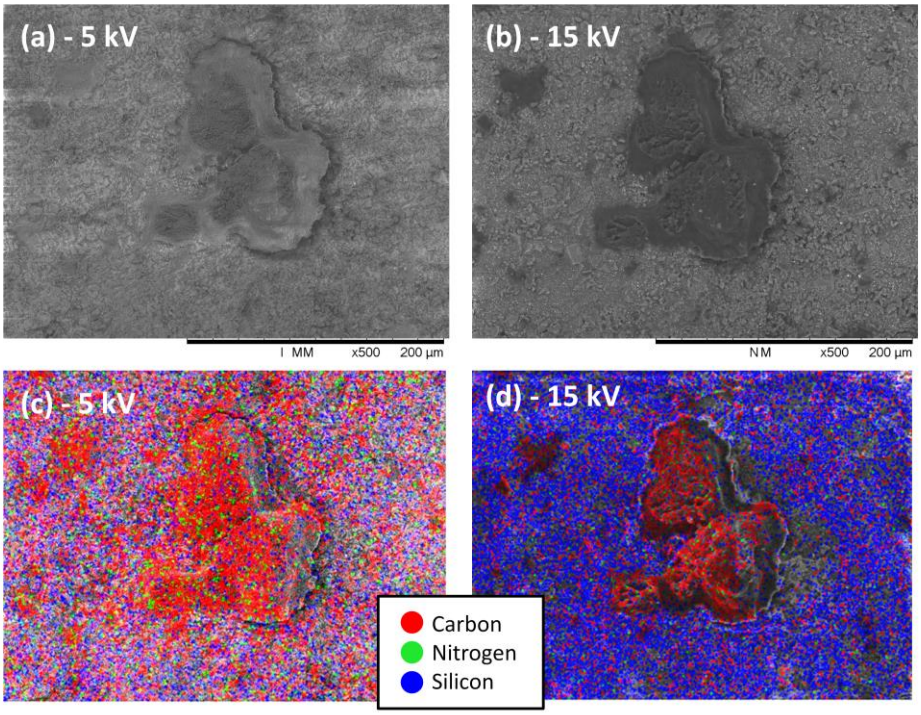
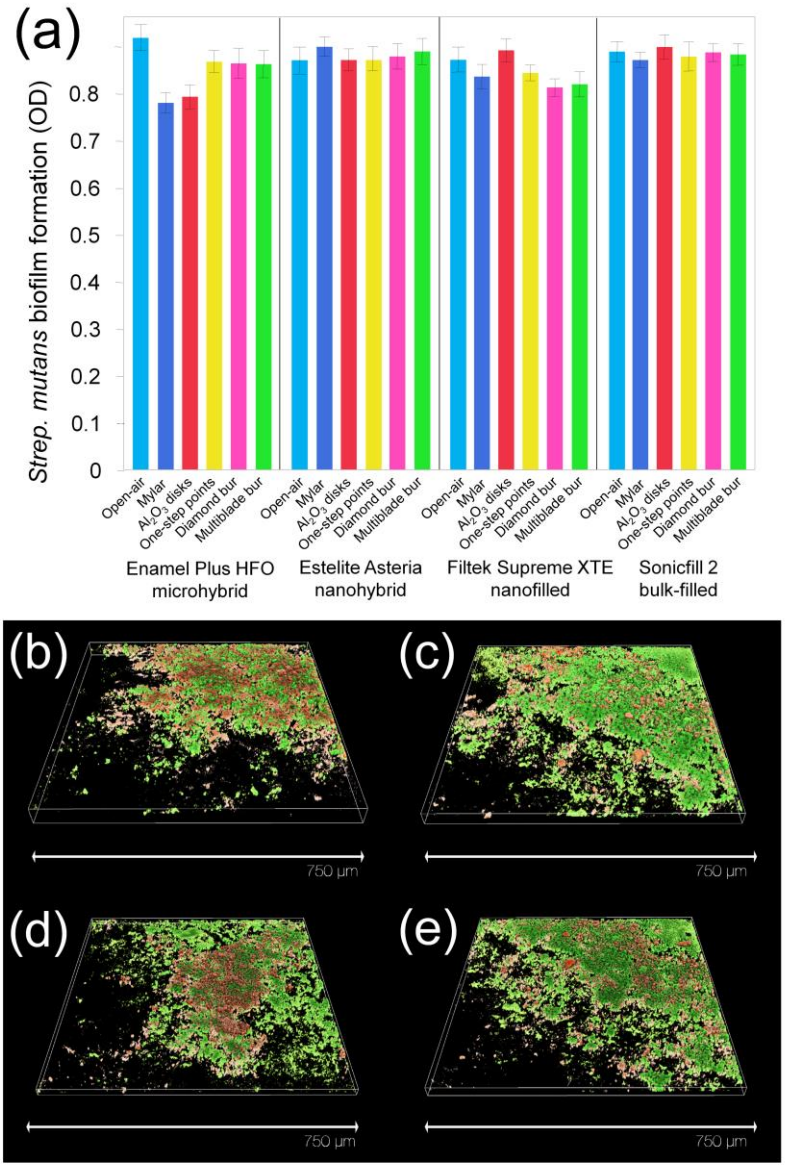


Fig. 5 *S. mutans* biofilm formation on the surface of the tested specimens. (a) shows results of viable biomass assay expressed as mean OD± SE. (b-e) are 3D reconstructions of the biofilms obtained by CLSM. LIVE/DEAD staining displays viable bacterial cells in green, while dead cells are shown in red. A representative field from specimens belonging to Group 3 is provided, as follows: (b) - microhybrid; (c) - nanohybrid; (d) - nanofilled; (e) - bulk-filled RBCs. No significant morphological differences in biofilm formation or in its viability were observed among the different F/P groups or RBC materials. All fields showed mature biofilm structures covering most part of the specimen surfaces, with a prevalence of viable cells.



Tables

Table 1. RBCs used in this study.

Type	Brand Name	Chemical composition	Manufacturer
Microhybrid	Enamel Plus HFO	Filler: 75 wt% 0.7 μm Sr, Al, silanized glass, 40 nm SiO_2 Base resin: Bis-GMA, UDMA, 1,4-butandioldimethacrylate	Micerium S.p.A., Avegno, Italy
Nanohybrid	Estelite Asteria	Filler: 82 wt% 200 nm supra-nano spherical filler SiO_2-ZrO_2, 200 nm composite filler (including spherical SiO_2-ZrO_2) Base resin: Bis-GMA, Bis-MPEPP, TEGDMA, UDMA	Tokuyama Dental, Tokyo, Japan
Nanofilled	Filtek Supreme XTE	Filler: 78.5 wt% 20nm SiO_2, 4–11nm ZrO_2, aggregated 0.6–1.4 μm SiO_2-ZrO_2 cluster Base resin: Bis-GMA, Bis-EMA, UDMA, TEGDMA, PEGDMA	3M, Maplewood, MN, USA
Bulk-filled	Sonicfill 2	Filler: 81.3 wt% SiO_2, barium glass, unreported filler size Base resin: 3-trimethoxysilylpropyl methacrylate, Bis-EMA, bisphenol-A-bis-(2-hydroxy-3-methacryloxypropyl) ether, TEGDMA.	Kerr Corporation, Orange, CA, USA

Bis-GMA, bisphenol-A-glycidyl-dimethacrylate; Bis-EMA, ethoxylated bisphenol-A-dimethacrylate; Bis-MPEPP, bisphenol-A-polyethoxy-methacrylate; UDMA, urethane dimethacrylate; TEGDMA, triethylene glycol dimethacrylate; PEGDMA, polyethylene glycol dimethacrylate.

Table 2. Mean composition (%wt) of the superficial layer (≈1μm) of the tested RBCs after light-curing against Mylar strips, as assessed by quantitative full-frame EDS. The sum of the elements composing the inorganic fraction is shown.

RBC	C (organic fraction)	Si	Al	Zr	F	Br	Ba	Yb	Na	W	Inorganic fraction
Microhybrid	**30.37**	14.58	3.43	0.00	0.00	0.00	0.49	0.00	0.00	11.41	**29.92**
Nanohybrid	**29.06**	20.48	0.00	10.45	0.00	0.00	0.00	0.00	0.58	0.00	**31.51**
Nanofilled	**22.62**	21.80	0.00	13.02	0.00	0.00	0.00	0.00	0.00	0.00	**34.82**
Bulk-filled	**36.55**	16.52	0.00	3.29	0.87	1.56	3.50	2.78	0.00	0.00	**28.51**