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Investigation of Bacterial Adhesion on Nanoparticle Filler-Reinforced Dental Composites after Different One-Step Finishing Timing Using a Constant-Depth Film Fermenter

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Abstract

This *in vitro* study evaluates the influence of application time of a one-step finishing system has on biofilm accumulation over nanoparticle filler-reinforced dental composites using a microcosm biofilm model in a constant depth film fermenter (CDFF). For that, sixty disk-shaped specimens ($\phi=5$ mm \times 2 mm thick) were made with nanoparticle filler reinforced dental composites (EsthetX™ and IPS Empress Direct). The specimens were manually polished with a finishing system (Enhance®) following the manufacturer's instructions for 5, 15 and 30 seconds, while unpolished specimens were used as controls. Oral biofilm was formed on the discs, using human saliva as inoculum and daily subject to 8 pulses of 10% sucrose solution. The biofilm was collected to determine the counts of total microorganisms. Data were analyzed by ANOVA-Tukey tests ($\alpha=5\%$). Increased time application of the finishing system significantly affected biofilm formation for tested materials ($p \leq 0.05$). Biofilm formation over the nanoparticle filler-reinforced dental composites was greatly reduced after 15 s of application. No additional significant reduction in biofilm was observed when the specimens were finished for 30 seconds.

However, differences related to the tested nanoparticle filler-reinforced dental composites were not statistically significant. By incorporating a minimum 15 s-time protocol into their everyday practices, dentists can positively reduce biofilm formation on nanoparticle filler-reinforced dental composites and contribute to the long-term esthetic and integrity these restorations.

Keywords: Oral biofilm; Composites; Nanoparticles; CDFF; Polishing

Introduction

In the last 20 years, nanotechnology has impact Dentistry in different aspects among different dental areas [1]. The field of direct restorative dental materials with special attention to composites have greatly captured the transition of emerging technology to actual dental practice [2]. Now, the vast majority of posterior restorations is performed using nanoparticle filler-reinforced dental composites since nanotechnology has led to the redesign of formulations for the current dental composite used by dentists for cavity fillings. Nanofillers are considered nanoparticles, approx. 100 nm, that are incorporated into the various composite composition as the inorganic filler. The advantage of nanofillers lay on creating composites with improving properties mainly for advances in mechanical and physical properties [3,4]. The filler size driven by nanotechnology had overcome the drawbacks of traditional fillers such micro filler and fibers and have targeted challenges like polymerization shrinkage, wear resistance, translucency, and roughness [5]. In this way, nanoparticle filler-reinforced dental composites present 50% to 78% of a mixer of spherical oxide (100 nm) and barium glass (400 nm) as filler content. This overall filler content influences the outcome results showing a material with highly polish ability properties and high surface luster.

When performing direct restorations, one of the main goals of dentists is to provide restorations with the high predictability of long-term clinical service [6]. The failure or success regarding clinical longevity of composite restorations depends on several extrinsic and intrinsic variables [7,8]. Operator, a method of isolation, and materials' composition including finishing/polishing are well-known crucial factors for the final satisfactory restorative outcome [9,10]. One of these relevant factors for improvement in the longevity of these materials is to obtain appropriated smoothness in the surface/interface of these restorations [11]. The set of clinical procedures, such as contouring, finishing and polishing steps to reach an appropriated smoothness rely on the chairside polishing protocol used by the operator [12]. In general, finishing/polishing procedures leads to surface roughness that

is undetectable by patient's tip tongue and presents a pleasant appearance [13]. For that, it has been proposed "one-step" polishing systems, polishing approach that meets the clinical demand for achieving a smooth surface within a minimum amount of time using a single instrument. However, obtaining a satisfactory polished surface is not only imperative for aesthetics, but it is also a key factor in oral biofilm formation over nanoparticle filler-reinforced dental composites [14].

The presence of nanofillers in the inorganic content of nanoparticle filler-reinforced dental composites and their differentiated characteristics may be reflected on alterations in oral biofilm over this class of materials since surface roughness has a great impact on plaque accumulation [15]. Nanoparticle filler-reinforced dental composites claimed to combine acceptable mechanical strength with optimal polishing/optical properties after being submitted to one-step polishing [16]. Therefore, the main objective of the present study was to evaluate the biofilm accumulation over nanoparticle filler-reinforced dental composites when submitted to the different timing for a one-step polishing.

Table 1 Commercial resins suggested to this study.

Resin	Classification	Manufacturer	Filler Content	Composition
EsthetX HD	Nanohybrid composite	light-cured DENTSPLY, Caulk, Milford, DE, USA	75.5%/54.60%	Bis-GMA adduct, a BisEMA adduct, and triethylene glycol dimethacrylate, Camphorquinone (CQ), photoinitiator, stabilizer, pigments, barium fluoroborosilicate glass below 1 µm and nanofiller silica (particle size 0.04 µm)
Empres s	Nanohybrid composite	light-cured Ivoclar Vivadent, Schaan, Liechtenstein, Germany	78.10%	0.4 µm barium glass filler, mixed oxide, Ba-Al- fluorosilicate glass (78.1%); dimethacrylate (21.5%), catalysts and stabilizers (0.4%), pigments (<0.1%)

The experimental units consisted of standardized nanoparticle filler-reinforced dental composite samples subjected to different surface treatments. The response variable was the mean CFU/mL present in the microcosm biofilm formed on the composite resin surface. Data were statistically analyzed by three-way analysis of variance (ANOVA) and the Tukey test ($p=0.05$).

Sample preparation

80-disc samples (5 mm Ø, 2 mm depth) of each nanoparticle filler-reinforced dental composites were prepared using cylindrical-shaped metal molds. The mold was filled in a single increment with the nanoparticle filler-reinforced dental composites. The top surface was cured for 40 seconds using a quartz-tungsten-halogen light curing unit (Optilux 501, Demetron/Kerr, Danbury, CT, USA) operating at 850 mW/cm².

One-step finishing timing

The samples of nanoparticle filler-reinforced dental composites were subdivided into four subgroups, each of which had 10 specimens, according to finishing as follows: T0; no finishing procedures were applied; T5: The samples were immediately finished per 5 seconds; T15: The samples were

Also, one of the most suitable methods of growing biofilms is the Constant Depth Film Fermentor (CDFF), since it allows the production of a stable oral biofilm community. The null hypotheses of the present study were that bacterial growth over nanoparticle filler reinforced dental composites will not be influenced by different one-step polishing timings.

Materials and Methods Experimental Design

This is *in vitro* study involved a 2×4 factorial design ($n=10$). The factors under evaluation were: Restorative materials at two levels the nanoparticle filler-reinforced dental composites Esthet XTM (Dentsply, Milford, DE, USA), and IPS Empress® Direct (Ivoclar Vivadent AG, Schaan, Liechtenstein) and One-Step Finishing System (Enhance, Dentsply Caulk) with time application at 4 levels: 0 seconds (control, 5 seconds (1 pass), 15 seconds (3 passes), and 30 seconds (6 passes). The composition and manufacturer of the composites tested are displayed in **Table 1**.

immediately finished per 15 seconds and T30: The samples were immediately finished per 30 seconds. The finishing procedures were performed using pre-mounted, single use, 40-µm aluminum oxide impregnated, cured urethane dimethacrylate resin finishers (Enhance® Finishers, type disc). The resin finishers designed for preparing composite surfaces for their final polish were attached to a high-speed handpiece at 10,000 rpm.

To control the variability, one investigator, blinded to which material was being processed, performed all the finishing and polishing procedures in a randomized order. After this, all samples were sterilized via an ethylene oxide sterilizer (Anprolene AN 74i, Andersen, Haw River, NC), degassed for seven days, and then used for bacteria inoculation and biofilm testing.

In vitro biofilm model using CDFF

Dental plaque biofilms were initiated from stimulated human saliva collected from 10 healthy, consenting individuals who refrained from oral hygiene 24 h before saliva collection [17]. Saliva was pooled in equal volumes of each sample, and 30% glycerol will be added [18]. All samples were stored at -80°C until needed.

A constant depth film fermentor (CDFF) was used to model the situation which occurs *in vivo* during the consumption of sucrose-rich drinks an approach which has been previously used to expose dental plaque to a cariogenic challenge [19]. Briefly, the CDFF consists of a glass vessel with a stainless-steel end-plate, with ports of the entry of medium, sucrose (10%, 8 ×/day) and gas and another port for waste medium. The growth medium contained mucin (type II, porcine, gastric) at a concentration of 2.5 g/L; bacteriological peptone, 2.0 g/L; tryptone, 2.0 g/L; yeast extract, 1.0 g/L; NaCl, 0.35 g/L; KCl, 0.2 g/L; CaCl₂, 0.2 g/L; cysteine hydrochloride, 0.1 g/L; haemin, 0.001 g/L; vitamin K1, 0.0002 g/L, at pH 7 [20].

An overview of the experimental setup used in this study is shown in **Figures 1A and 1B**. The CDFF vessel housed 15 polytetrafluoroethylene sampling pans rotating under a polytetrafluoroethylene scraper bar that smeared the incoming medium over the pans and maintained the formed biofilms at a constant depth of 300 µm [21]. This movement simulates the mechanical biofilm removal promoted by human tongue inside the oral cavity and wipes off the excess of medium and bacterial suspension.

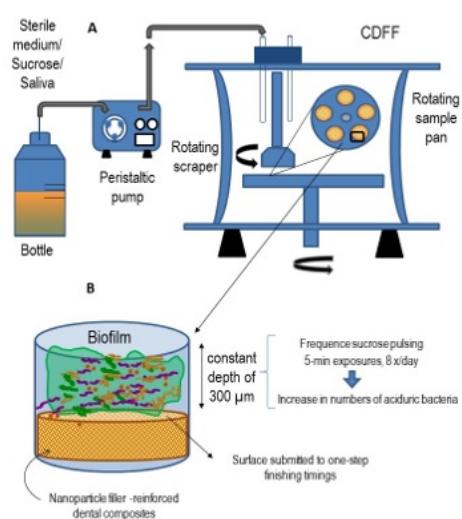


Figure 1 (A) Schematic drawing of the experimental setup using constant-depth film fermenter. **(B)** Close-up view of the oral biofilm over nanoparticle filler reinforced dental composites located in one of the wells of the polytetrafluoroethylene sampling pan.

Each sampling pan had five cylindrical holes ($\varnothing=5$ mm). The two groups of nanoparticle filler-reinforced dental composites subjected to the different one system finishing timing were placed into these cylinders at a depth of 300 µm below the upper surface of the pan (two sampling pans per group). After mounting, the CDFF was autoclaved at 121°C for 15 min [19].

To initiate the biofilm adhesion and formation over the nanoparticle filler-reinforced dental composites, saliva collected (10 ml) was added to 200 ml of sterile medium and pumped to the system (2.4 ml/min). One hour after initial inoculation, the sterile medium was pumped and supplied

dropwise, at a flow rate of 0.5 ml/min to induce and maintain biofilm growth after inoculation [17]. Sucrose pulsing was started four h after the initial inoculum. The protocol for sucrose challenge was 2.4 ml/min, 5-min exposures, 8 ×/day, with 2-hour interval between exposures and a daily 10-hour period for overnight rest [22].

Evaluation of bacterial viability

On the 5th day after inoculation, the biofilm growth over the discs was collected aseptically of the CDFF and put in microtubes of centrifuge containing 0.9% sodium chloride solution and dispersed by sonication [22]. Subsequently, aliquots of this suspension were plated in triplicate in brain heart infusion. The plates were incubated for 48 hours at 37°C in a partial atmosphere of 10% CO₂. The results were expressed in CFU/disc.

Microscopy fluorescent assay

For microscopic fluorescence visualization of the adherent biofilm over the samples, the disks were gently washed three times with phosphate buffered saline (PBS) and then stained using a live/dead bacterial viability kit (Molecular Probes, Eugene, OR). Live bacteria were stained with Syto 9 to produce a green fluorescence, and bacteria with compromised membranes were stained with propidium iodide to produce a red fluorescence. The stained discs were examined using an epifluorescence microscope (TE2000-S, Nikon, Melville, NY) [20].

Statistical analysis

All statistical analyses were performed using statistical software (Statistical evaluations were performed with SigmaStat 3.5 (Systat, San Jose, CA). The normality and homogeneity were checked for each variable. A two-way factorial ANOVA and a post hoc Tukey test performed setting the nanoparticle filler-reinforced dental composites at two levels and one-step finishing timing at four levels (T0, T5, T15 and T30) as fixed factors. The normality of error distribution and the degree of non-constant variance were checked for each response variable. The level of significance (α) was set to 0.05.

Results

The mean and respective standard deviations expressed in CFU/disc after the evaluated finishing times are plotted in **Figures 2A and 2B**. When the CFU counts in each studied nanoparticle filler-reinforced dental composites were compared among the four timings, the groups control (no polishing=T0) and the group subjected to 5 seconds presented similar biofilm growth. A two-way ANOVA found a significant effect for timing ($p<0.001$) but no significant effect for composite type ($p=0.81$) or for the interaction between these factors ($p=0.94$). The Tukey post hoc test ($p<0.05$) revealed the following significant differences: the nanoparticle filler-reinforced dental composites subjected to 5 seconds (T5)

presents the highest biofilm growth which was similar to control (T0) group. The lowest CFU/disc was found for T15 and T30 groups, which were statistically similar to each other, but different from control=T0 and T5 groups.

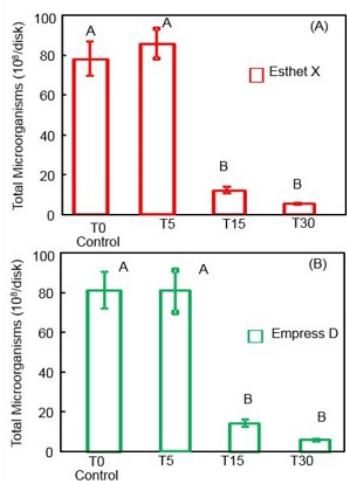


Figure 2 (A) Means \pm SD of the total microorganism's colony-forming unit (CFU) counts per disc according to the one step polishing timing for the nanoparticle filler-reinforced dental composite EsthetX and **(B)** Means \pm SD of the total microorganism's colony-forming unit (CFU) counts per disc according to the one step polishing timing for the nanoparticle filler-reinforced dental composite Empress Direct. All CFU counts on both nanoparticle filler-reinforced dental composites submitted to 15 seconds or 30 seconds timing were more than four folds lower than those submitted to 5 seconds timing or not finished. Groups identified by different capital letters represent statistically significantly difference among the timing ($p<0.05$).

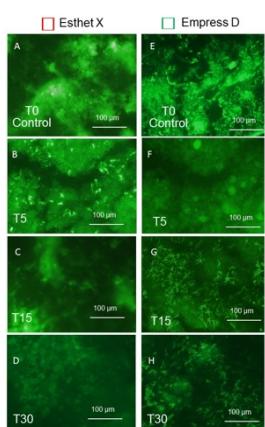


Figure 3 Typical fluorescence microscopic images demonstrating biofilm growth over the nanoparticle filler-reinforced dental composites. **(A-D)** and **(E-H)** show the biofilm accumulation over Esthet X and Empress D, respectively.

In **Figure 3**, typical fluorescence live/dead images of 5-day biofilms on nanoparticle filler-reinforced dental composites. All the samples were fully covered by primarily live bacteria. **Figures 3A-3D** and **Figures 3E-3H** show the biofilm accumulation over Esthet X and Empress D®, respectively. For control and the timing of 5 seconds their massive growth with a dense layer of biofilms considering both tested nanoparticle filler-reinforced dental composites. After one-Step Finishing timing of 15 seconds, both materials had less biofilm accumulation.

Discussion

Biofilm formation over nanoparticle filler-reinforced dental composites can lead to a negative spiral of events resulting in the development of caries around or below a restoration [23]. Insufficient finishing can cause increased biofilm accumulation, which compromises the clinical performance of the restoration. Nanoparticle filler-reinforced dental composites compromise in its inorganic composition a mixed of nanoparticle and its influence on biofilm formation facing different times using one-step finishing system was investigated in this study. Previous studies state that biofilm adhesion varies according to the to the filler size of reinforcement particles since nano sized particles reduce roughness after finishing procedures and consequently decreasing bacterial adherence [24,25]. The results of this study aimed to contribute to understanding the relationship between nano-filler size and polishing timing, as well as other factors associated with caries development, such as biofilm growth and accumulation over the most widely used direct restorative dental materials: nanoparticle filler-reinforced dental composites.

In attempts to understand better the behavior of nanoparticle filler-reinforced dental composites during the process of bacterial adhesion and biofilm formation over the material, and to observe a more clinically relevant outcome, the specimens were submitted to constant-depth film fermenter [17,22]. The selection of the biofilm model using CDFF is based on the fact this methodology allows to assess the biofilm developed under low shear forces. The main characteristic feature is the z (depth)-restriction of the cultivated biofilms by mechanical removal of excess biofilm with a scraper simulating the abrasive movement of the tongue [26]. This feature associated to variables related to the environment, such as saliva, pH and temperature simulates a more realistic clinical environment.

According to our results, the null hypothesis stating no difference in biofilm accumulation over nanoparticle filler-reinforced dental composites when subjected to different timing was rejected. The use of one-step finishing for 15 seconds was able to show values statistically different from control group. Findings from this study demonstrated that the finishing procedures should be done for at least 15 seconds to reach a smoothness able to promote less biofilm accumulation over nanoparticle filler-reinforced dental composites. The fluorescence images of nanoparticle filler-reinforced dental composites after 15 seconds also support the finding. This

result assumes that surfaces, where the increased duration of finishing procedure were performed, are less favorable for biofilm formation than those with short duration. An explanation of this phenomenon can be related to the exposure of particles, such as silicon dioxide and barium fluorosilicate, which are extremely small and irregular, improving the quality of roughness, and lowering the contact angles for bacterial adhesion [27].

Currently, only a few studies with conflicting results have determined the biofilm growth behavior over nanoparticle filler-reinforced dental composites considering finishing timing. Previous study [28,29] has positively correlated roughness with duration of the finishing procedure using the same polishing system, where significantly less roughness surface was found for 30 seconds timing.

In relation to the composition of the studied material, the tested nanoparticle filler reinforced dental composites presented similar inorganic content. The literature suggests that filler size has the potential to influence the surface characteristics of composite [30-32]. This may suggest that nano-filler incorporation response to finishing timing were similar since the ability to produce a smooth surface with the use of the aluminum oxide disks depends on their cutting filler particles and matrix resin equally [33,34]. Since the nanoparticle filler-reinforced dental composites used in this study were highly filled hybrid composites with relatively large filler particles, further investigation focusing specifically in high content nanofiller is a need.

Conclusion

Results suggest that to achieve long-lasting esthetics in nanoparticle filler-reinforced dental composites, special attention should be paid to finishing timing. By incorporating a minimum 15 s-time protocol for the use of the one step finishing system used in this study, dentists can reduce biofilm formation and contribute to the long-term esthetic and biofilm-resistant predictability of nanoparticle filler-reinforced dental composites.

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