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Original Article

Effect of brushing on surface roughness, fluoride release, and biofilm formation with different tooth-colored materials

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Biofilm formation;
Brushing;
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Abstract *Background/purpose:* Tooth brushing, material mechanical ageing procedure, is the most effective way in removing biofilm. The purpose of this study was to investigate the surface roughness, fluoride-release, and S. mutans biofilm formation on various tooth-colored restorative materials before and after brushing.
Materials and methods: Discs of materials, a nanocomposite (Filtek Z350XT; CO), a giomer (Beautifil II; GIOMER), a resin-modified glass-ionomer material (Fuji II LC; RMGI), and a conventional glass-ionomer material (Fuji IX GP Extra; GI), were prepared, polished with abrasive discs (SofLex), and divided into brushed and not brushed groups. The surface roughness of specimens was observed using a contact profilometer, fluoride-release was measured using a fluoride-specific ion electrode, and S. mutans biofilm formation, biovolume and live/dead cells, was observed under a confocal laser scanning microscope.
Results: Higher roughness was observed on GI and RMGI than on CO and GIOMER. Brushing had no effect on the roughness. The fluoride-release of GI and RMGI was higher than that of GIOMER. The fluoride-release decreased after brushing in all materials. The biovolume of S. mutans was not significantly different between GIOMER, RMGI and GI, while CO showed the highest. Brushing resulted in a higher biovolume for all materials, except CO, which showed no change. After brushing, all the tested materials demonstrated identical biovolumes. There were no significant differences in live/dead cells among all groups.

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Conclusion: Brushing demonstrated a negative effect on the fluoride-release and biovolume of S. mutans biofilms for all tested materials except nanocomposites.

Introduction

There are several tooth-colored restorative materials available on the dental market, including conventional glass-ionomer material, resin-modified glass-ionomer material, polyacid-modified resin composite, giomer and resin composite. The main differences among these materials are their chemical compositions and physical and mechanical properties. Glass-ionomer materials are well known as the best fluoride-releasing and fluoride-recharging materials, but they have low mechanical properties.[1–3] However, the recharging process depends on recharging agents and their frequency of exposure. One of the most common fluoride-recharging process are the brushing with fluoridated toothpaste.[4] While resin composites are well known as highly esthetic materials that have good mechanical properties but less fluoride release.[1,5,6] This has led to the development of materials such as resin-modified glass-ionomer materials, polyacid-modified resin composites and giomers that contain properties of both glass-ionomer materials and resin composites to provide better levels of fluoride release than resin composites and better mechanical properties than glass-ionomer materials.[7–11]

The finishing and polishing processes of dental restorations are important steps for achieving longevity and aesthetics. Different materials present different mechanical properties and different filler loadings, which can result in a nonuniform abrasion level after the polishing process.[12,13] Surface properties of dental materials, such as the surface roughness and chemical compositions, influence biofilm retention. Many studies have shown that the rougher the surface is, the more the biofilms and bacterial adherence can occur.[13–16] Biofilms are reported to be associated with common oral diseases, such as dental caries and periodontal diseases.[17,18]

A very simple and common method used in the prevention of common oral diseases is tooth brushing. General recommendations by American Dental Association in 2017 are to brush twice a day with an optimal duration of 2 min per whole mouth using fluoridated toothpaste associated with significantly reduction in biofilm for reducing risk of caries and periodontitis.[19] Thus, tooth brushing may result in an increase in the surface roughness of both enamel and existing restorations that leads to more biofilm adherence.[13,16,20–22]

The purpose of this study was to investigate *Streptococcus mutans* biofilm formation on various types of tooth-colored restorative materials both before and after brushing simulation.

Materials and methods

This study was performed using protocols approved by the Faculty of Dentistry/Faculty of Pharmacy, Mahidol University Institutional Review Board (COE 2017/010.3003).

Four commercially available tooth-colored restorative materials were used in this study: nanocomposite (Filtek Z350XT, 3M ESPE, St. Paul, MN, USA), giomer (Beautifil II, Shofu Inc., Kyoto, Japan), resin-modified glass-ionomer material (GC Fuji II LC Capsule, GC Corporation, Tokyo, Japan) and conventional glass-ionomer material (GC Fuji IX GP Extra Capsule, GC Corporation.). The details of the materials are shown in Table 1. GC Fuji II LC capsules and GC Fuji IX GP extra capsules were activated and mixed with a capsule mixer (Silamat S6, Ivoclar Vivadent AG, Schaan, Liechtenstein) at 4500 rpm for 10 s.

Cylindrical-shaped specimens of each material were prepared using a plastic tube with a diameter of 5 mm and height of 2 mm. The material was placed into a plastic tube, and both sides of the tube were covered with polyester celluloid strips (Stripmat, Polydentia, Mezzovico, Switzerland) and then covered again with glass slides. Constant pressure was applied to the glass slides. For the nanocomposite resin, giomer and resin-modified glass-ionomer materials, each side of the specimens was light-cured for 20 s using a light-curing unit (Bluephase New, Ivoclar Vivadent AG) with the curing tip against the glass slide. The conventional glass-ionomer material was allowed to self-cure for 2 min and 20 s. Resin-modified glass-ionomer material and conventional glass-ionomer material were immediately coated with a nanofilled resin coating (EQUIA Coat, GC Europe, Leuven, Belgium) and light-cured for 20 s using a light-curing unit. All specimens were kept in 100% relative humidity at 37 °C, and after complete polymerization for 24 h, the specimens underwent surface polishing with 4 sequences of abrasive polishing discs (Sof-Lex extra thin, 3 M ESPE). Four different polishing directions were used with a summation of 60 s per sequence using a speed-controlled handpiece (TCM ENDO III, SybronEndo, Nouvag AG, Switzerland) running at 13,000 rpm under dry conditions. Seven polishing strokes were performed by one operator for 15 s in each direction with a constant pressure of approximately 2 N. The specimens were rinsed with water and blown with air between each polishing sequence. Each abrasive polishing disc was used with only one specimen. The polished specimens were cleaned with ultrasonication (BioSonic UC125, Coltene Whaledent, Altstatten, Switzerland) in distilled water for 5 min and then allowed to air dry.

Table 1 Materials' details according to manufacturer's data.

Materials	Type	Compositions	Mean particle size of filler	Percent of filler by volume	Percent of filler by weight	Manufacturer
Filtek Z350 XT	Nanocomposite	**Resin matrix**: Bis-GMA, TEGDMA, UDMA, Bis-EMA **Filler**: silica nanofillers, zirconia/silica nanocluster	20 nm (nanofillers) 0.6–1.4 μm (nanocluster)	55.6	72.5	3M ESPE, St. Paul, MN, USA
Beautifil II	Giomer	**Resin matrix**: Bis-GMA, TEGDMA **Filler**: Multifunctional glass filler and S-PRG filler based on fluoroboroaluminosilicate glass	0.8 μm (0.01–4 μm)	68.8	83.3	Shofu, Kyoto, Japan
GC Fuji II LC Capsule	Resin-modified glass-ionomer material	Liquid: Distilled water, Polyacrylic acid, HEMA, UDMA, Comphorquinone Powder: Fluoroaluminosilicate glass	5.9 μm	–	–	GC Corporation, Tokyo, Japan
GC Fuji IX GP Extra Capsule	Conventional glass-ionomer material	Liquid: Distilled water, Polyacrylic acid Powder: Fluoroaluminosilicate glass, Polyacrylic acid powder	10 μm	–	–	GC Corporation, Tokyo, Japan

(Bis-GMA: Bisphenol A diglycidyl methacrylate, TEGDMA: Triethyl glycol dimethacrylate, UDMA: Urethane dimethacrylate, Bis-EMA: Ethoxylated bisphenol A dimethacrylate, S-PRG: Surface prereacted glass-ionomer, HEMA: Hydroxyethyl methacrylate).

Fifty discs of each material were randomly divided and subjected to investigation in 3 parts; 20 discs were used to determine the surface roughness, 10 discs were used to determine the amount of fluoride released, and 20 discs were used in the biofilm formation assay.

For brushing simulation, half of the prepared specimens of each material were subjected to simulated brushing with a GUM classic toothbrush 311 (Sunstar Americas, Schaumburg, IL, USA) and Colgate anticavity fluoride toothpaste (Colgate anticavity fluoride toothpaste, Colgate-Palmolive, Chonburi, Thailand) using a brushing machine (TBS-V8, King Mongkut's Institute of Technology, Bangkok, Thailand) with a load of 200 g[23] at a frequency of 100 strokes per minute for 20,000 strokes to represent 2 years of brushing twice per day.[24] The specimens were placed into the brushing machine chamber containing a slurry of 25 g of dentifrice and 40 ml of deionized water.

Determination of surface roughness

Ten specimens of each material from both the no brushing and brushing groups were examined to determine the average absolute roughness (Sa) by 201 parallel tracing at the center 3 × 3 mm^2 area under a contact profilometer with a 2.0 μm tip radius stylus (TalyScan 150, Taylor Hobson LTD, Leicester, UK) with a speed of 1500 μm/s to give a 3D reconstructed image. The data were then filtered with a cutoff of 0.08 mm (Gaussian profile filter). One recording was made for each specimen. The surface roughness parameter of Sa was calculated by Talymap 3D analysis software (Taylor Hobson LTD).

Amount of fluoride released

Five specimens of each material from both the no brushing and brushing groups were subjected to fluoride release tests. The specimens were placed in 24-well culture plates, immersed in 1 ml of deionized water and incubated in a 5% CO_2 chamber at 37 °C for 24 h. The specimens were removed, and the sample solution was transferred into a clean plastic tube and mixed with 100 μl TISAB III (total ionic strength adjustment buffer, 940,911, Thermo Scientific Orion, Beverly, MA, USA). The solution was used to measure the amount of fluoride released three times from each material disc using a fluoride-specific ion electrode (Orion EA940 expandable, Orion Research, Beverly, MA, USA) connected to an ion analyzer (Orion ion analyzer EA940, Orion Research). The fluoride electrodes can detect fluoride concentrations as small as 0.02 part per million (ppm). The mean fluoride concentration in ppm of each specimen was calculated and used as representative data for each specimen.

Determination of biofilm formation

Ten specimens of each material from both the no brushing and brushing groups were subjected to biofilm formation tests using a confocal laser scanning microscope (CLSM) (Fluoview 10i; Olympus Corporation, Tokyo, Japan). The specimens were sterilized in a UV chamber for 1 h and 30 min per side prior to biofilm formation assays under CLSM.[11,25]

Unstimulated saliva was collected from 3 healthy donors who had no medical problems and no medicine intake within 1 month under protocols approved by the ethical committee. Saliva was centrifuged at 4000 g at 4 °C for 15 min. The centrifuged saliva was pooled and diluted in phosphate buffer solution (PBS) at a ratio of 1:10, underwent a sterilization process using filtration devices at a pore size of 0.2 μm and kept at 4 °C until use.

The cariogenic bacteria *S. mutans* (ATCC 25175, Thai Can Biotech, Bangkok, Thailand) were cultured in brain-heart infusion agar (BHI) in a 5% CO_2 incubator at 37 °C for 48 h. Bacteria were then cultivated in brain-heart infusion broth supplemented with 5% sucrose in 5% CO_2 at 37 °C to achieve the desired turbidity at a cell density of 1×10^8 CFU/ml or 0.5 McFarland.

Ten saliva-coated specimens from each group were prepared by immersing each specimen in 1 ml filtrated saliva in a 24-well culture plate for 16 h at 37 °C to form an acquired pellicle. Saliva-coated specimens were then immersed and incubated with 1 ml of bacterial suspension in a 5% CO_2 chamber at 37 °C for 24 h. After incubation, the specimens were rinsed with 1 ml distilled water 3 times and transferred to a clean 24-well culture plate. Five specimens from each group were screened to determine whether a biofilm was formed by crystal violet staining, and they were observed under a stereomicroscope (Leica EZ4 HD, Leica Microsystems, Wetzlar, Germany). The biofilm formation on the remaining 5 specimens was tested to determine the biovolume and the live and dead cell ratio using CLSM (Fluoview 10i, Olympus Corporation).

For CLSM observation, the specimens were stained using a Live/Dead Bac Light™ Bacterial Viability kit (Molecular Probes, Eugene, OR, USA) in 24-well plates. This kit is composed of two fluorescent nucleic acid dyes: SYTO 9 and isopropidium iodide. SYTO 9 stains the cells green, as it can penetrate bacterial cell membranes. Isopropidium iodide stains only dead cells, as it can penetrate only cells with damaged membranes, and combining the two stains produces a red color. The dyes were diluted separately with filtered distilled water by mixing 3 μl of each dye into a tube containing 1 ml of filtered distilled water. The mixed dyes were then placed onto a specimen in which a biofilm was formed and soaked for 20 min under light protection.

Three neighboring points at the center of each specimen were analyzed under CLSM. An excitation wavelength of 488 nm was used, and the emitted light was collected between 500 and 560 nm. The specimens were observed using optical lenses with a magnification of 60x. The sectional images were then reconstructed to a 3-dimensional model using FV 10-ASW software (V 1.7a, Olympus Corporation). The biovolume, the bacterial density over a studied area (45,000 μm^2), was calculated using the color segmentation method. The live and dead numbers of *S. mutans* cells in the biofilms were calculated from the total number of green and red pixels from FV 10-ASW software.

Statistical analysis

Statistical analysis was performed using PASW statistics 18 (SPSS Inc., Chicago, IL, USA). The means and standard deviations of all the groups were calculated. Two-way ANOVA was used to examine the effect of brushing and the effect of the tested materials on the absolute average roughness, amount of fluoride released, biovolume of biofilms and bacterial live/dead cell ratio. Post hoc tests were performed using Tukey's multiple comparison for biovolume and live/dead cell evaluation and Dunnett T3 multiple comparison for average absolute roughness measurement and amount of fluoride released. Statistical analysis was performed with a level of significance *p-value* of 0.05.

Results

Average absolute roughness measurement

According to 2-way ANOVA, both tested materials and brushing conditions had an effect on the average absolute roughness ($p < 0.01$). The interaction between two factors was found with $p = 0.024$. Means and standard deviations of average absolute roughness from 4 tested materials with simulated brushing conditions are shown in Table 2. The mean Sa values of each material that underwent brushing were not significantly different from those of the no brushing groups. Sa values between Fuji II LC and Fuji IX GP Extra were not significantly different but were significantly higher than those of Z350 XT and Beautifil II. The Sa values of Z350 XT and Beautifil II were also not significantly different ($p > 0.05$). Representative 3D images showing the surface topography of all the groups are shown in Fig. 1. Intact and relatively smoother surfaces of Z350 XT and Beautifil II were observed in both the no brushing and brushing conditions. The images of Fuji II LC and Fuji IX GP Extra demonstrated generalized surface irregularities.

Amount of fluoride released

Significant effects of the tested materials ($p < 0.01$) and brushing condition on the amount of fluoride released ($p < 0.01$) and the interaction between the tested materials and brushing ($p < 0.01$) were found by 2-way ANOVA. The means and standard deviations of the amount of fluoride released from the 4 tested materials under simulated brushing conditions are shown in Table 3. Fuji II LC and Fuji IX GP Extra demonstrated the highest amount of fluoride released under brushing conditions, followed by Beautifil II and Z350 XT. The amount of fluoride released significantly decreased after brushing for all the tested

Table 2 Absolute roughness (μm) (means ± standard deviation) of tested materials.

Materials	No brushing	With brushing
Z350 XT	$0.08336 \pm 0.01303^{d,e,f}$	$0.08113 \pm 0.01098^{e,f}$
Beautifil II	$0.09145 \pm 0.01627^{d,e,f}$	$0.11064 \pm 0.01422^{d,e}$
Fuji II LC	$0.28056 \pm 0.04424^{a,b,c}$	$0.34041 \pm 0.03544^{a,b}$
Fuji IX GP Extra	$0.23132 \pm 0.04310^{b,c}$	$0.26153 \pm 0.02637^{b,c}$

The data with the same superscript letter demonstrate no statistically significant difference.

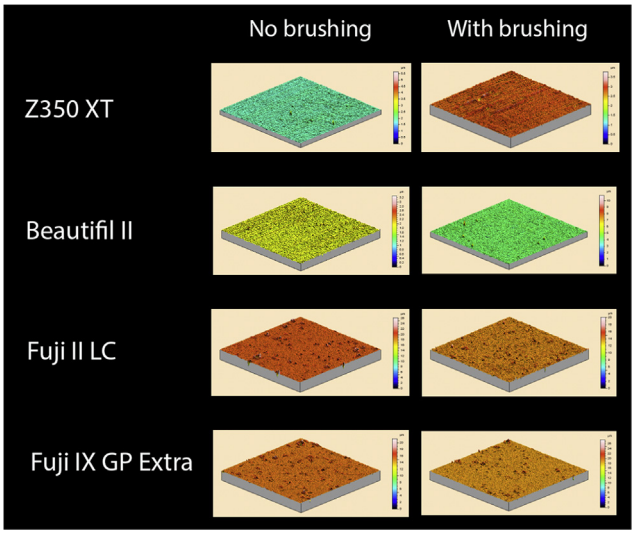

Figure 1 Representative 3D images from the Talmap 3D analysis software of tested materials.

Table 3 Amount of fluoride released (ppm) (means ± standard deviation) from the tested materials.

Materials	No brushing	With brushing
Z350 XT	0.0032 ± 0.0015^{e}	0.0040 ± 0.0026^{e}
Beautifil II	1.0600 ± 0.0189^{c}	0.4378 ± 0.0465^{d}
Fuji II LC	13.3296 ± 0.6336^{a}	7.8011 ± 0.4851^{b}
Fuji IX GP Extra	19.4295 ± 1.8697^{a}	8.2911 ± 0.5174^{b}

The data with the same superscript letter demonstrate no statistically significant difference.

materials ($p < 0.05$) except Z350 XT, which remained unchanged ($p > 0.05$).

Biovolume

From 2-way ANOVA, both the materials tested and brushing conditions had a significant effect on the biovolume of *S. mutans biofilms* ($p < 0.01$). The interaction between two factors was found with $p < 0.01$. The means and standard deviations of biovolumes from the 4 tested materials with simulated brushing conditions are shown in Table 4. Without brushing, the biovolume of *S. mutans* on Z350 XT was statistically higher than that on Beautifil II, Fuji II LC and Fuji IX GP Extra. With brushing, the biovolumes were not significantly different among all the tested materials. When comparing each type of material, brushing resulted in a statistically higher biovolume in Beautifil II, Fuji II LC and Fuji IX GP Extra. Z350 XT demonstrated no statistically significant difference ($p = 0.21$). The representative images of biovolume or cell densities are shown in Fig. 2. Biofilm staining was performed with a live/dead BacLight bacterial viability kit. Live and dead cells are shown in green and red, respectively. The combinations between live and dead cells are shown in yellow. Without brushing, the color intensity on Z350 XT indicates a biovolume of *S. mutans* greater than that of Beautifil II, Fuji II LC and Fuji IX GP Extra. On the other hand, in the brushing groups, there were no obvious differences in the color intensities between all materials. When conducting a comparison within each material, the brushing resulted in a greater intensity in Beautifil II, Fuji II LC and Fuji IX GP Extra, and Z350 XT had no difference.

Table 4 Biovolume ($\mu m^3\ \mu m^{-2}$) (means ± standard deviation) of *S. mutans* biofilms on the tested materials.

Materials	No brushing	With brushing
Z350 XT	0.13574 ± 0.02198^{a}	0.10733 ± 0.01511^{a,b}
Beautifil II	0.06877 ± 0.00918^{c}	0.10620 ± 0.01145^{a,b}
Fuji II LC	0.05899 ± 0.00950^{c}	0.11042 ± 0.02840^{a,b}
Fuji IX GP Extra	0.09185 ± 0.02082^{b,c}	0.13855 ± 0.01501^{a}

The data with the same superscript letter demonstrate no statistically significant difference.

S. mutans live/dead

The materials and brushing conditions were two main factors that were used to analyze the live/dead ratios. Statistical analysis using 2-way ANOVA revealed that both materials ($p = 0.13$) and brushing conditions ($p = 0.42$) had no effect on the live/dead ratios of *S. mutans* biofilms, and no interaction between factors was found ($p = 0.06$). Means and standard deviations of the cell live/dead ratio from the 4 tested materials with 2 simulated brushing conditions are shown in Table 5. No significant differences were found among all groups. The representative images of live/dead cell staining are shown in Fig. 3. The green (live cells) and red (dead cells) intensities were consistent with the biovolume, and they were lower in the nonbrushing groups of Beautifil II, Fuji II LC and Fuji IX GP Extra.

Discussion

Four commonly used tooth-colored restorative materials, nanocomposite, giomer, conventional glass-ionomer materials and resin-modified glass-ionomer materials, were used. Nanocomposites and giomers were used to represent resin-based materials, while conventional glass ionomer materials and resin-modified glass ionomer materials were used to represent glass ionomer-based materials. Therefore, studies using different the nanofilled composites and glass-ionomer materials available in the dental market might yield different outcomes.

The first part of the study was to evaluate the surface roughness of tested materials using three-dimensional (3D) surface roughness measurements. The average absolute roughness (Sa) parameter is an arithmetical mean height of an area measured from a 3D system that is more accurate in interpreting the average surface roughness of a whole surface.[26] Regarding the Sa measurement, the Sa was lower in the resin-based materials Z350XT than in the glass-ionomer-based materials. This result conformed with the study of Gladys and coworkers and Momoi and coworkers.[2,5] In this study, no significant difference in Sa was found within the material after brushing for 20,000 strokes, which mimicked 2 years of daily brushing.[24]

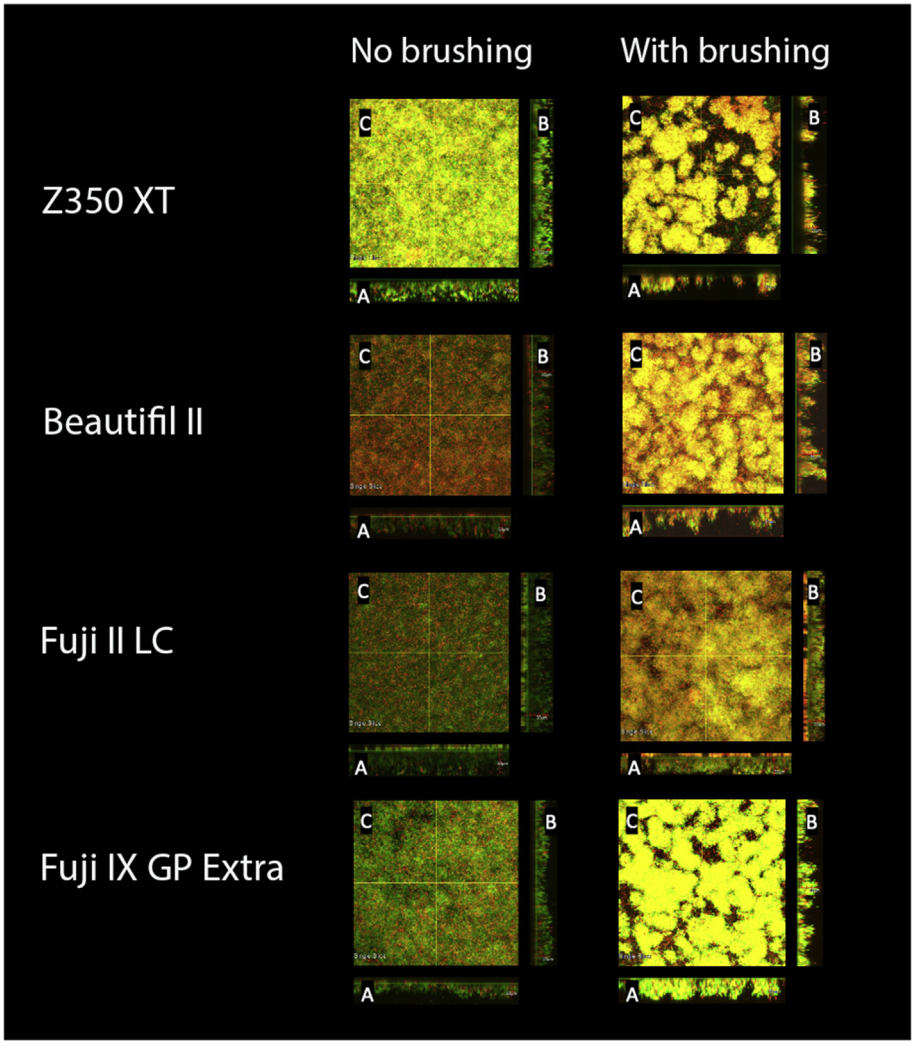

Figure 2 Representative images of *S. mutans* biovolume on different materials from CLSM using FV10-ASW software. A is a Z-projection in the X-Z direction, and B is a Z-projection in the Y-Z direction. The optically combined Z-stack slices are shown in C. The yellow lines in C indicate orthogonal planes of the X-Z and Y-Z projections, respectively.

Table 5 *S. mutans* live/dead ratios (means ± standard deviation) in biofilms on the tested materials.

Materials	No brushing	With brushing
Z350 XT	1.3168 ± 0.2556^{a}	0.8579 ± 0.2897^{a}
Beautifil II	0.7280 ± 0.3618^{a}	0.9687 ± 0.4600^{a}
Fuji II LC	1.0299 ± 0.3333^{a}	1.2718 ± 0.4704^{a}
Fuji IX GP Extra	1.4451 ± 0.5349^{a}	1.0360 ± 0.1345^{a}

The data with the same superscript letter demonstrate no statistically significant difference.

According to the 3D images of surface topographies, Z350XT and Beautiful II showed intact and smoother surfaces. Their surface characteristics differed completely with those observed with the Fuji II LC and Fuji IX GP Extra, in which generalized surface irregularities were found. Materials that either consist of a larger number of surface irregularities or contain a larger particle commonly show higher surface roughness.[5] In this study, various particle size materials were used; Z350XT, Beautiful II, Fuji II LC and Fuji IX GP Extra have mean particle sizes of 20 nm, 0.8 μm, 5.9 μm and 10 μm, respectively. To mimic clinical steps in finishing and polishing tooth-colored restorative materials, four complete sequences of Sof-Lex abrasive discs were used to polish all specimens. The matrix of glass-ionomer-based materials was composed of loosely bound cation cross-linked polyacid molecules rather than the durable bond-like silane coupling agents that were used in resin-based material manufacturing. The use of coarse grit abrasive discs might have led to the dislodgement of larger particles from the matrix of glass ionomer-based materials such as Fuji II LC and Fuji IX GP Extra. Consequently, surface irregularities and higher surface roughness were found in these two glass ionomer-based materials. However, using encapsulated glass-ionomer material leads to a lower number of larger diameter porosities compared with hand-mixed cements. This study still proves that the mechanical mixing process leads to the formation of pores in contrast

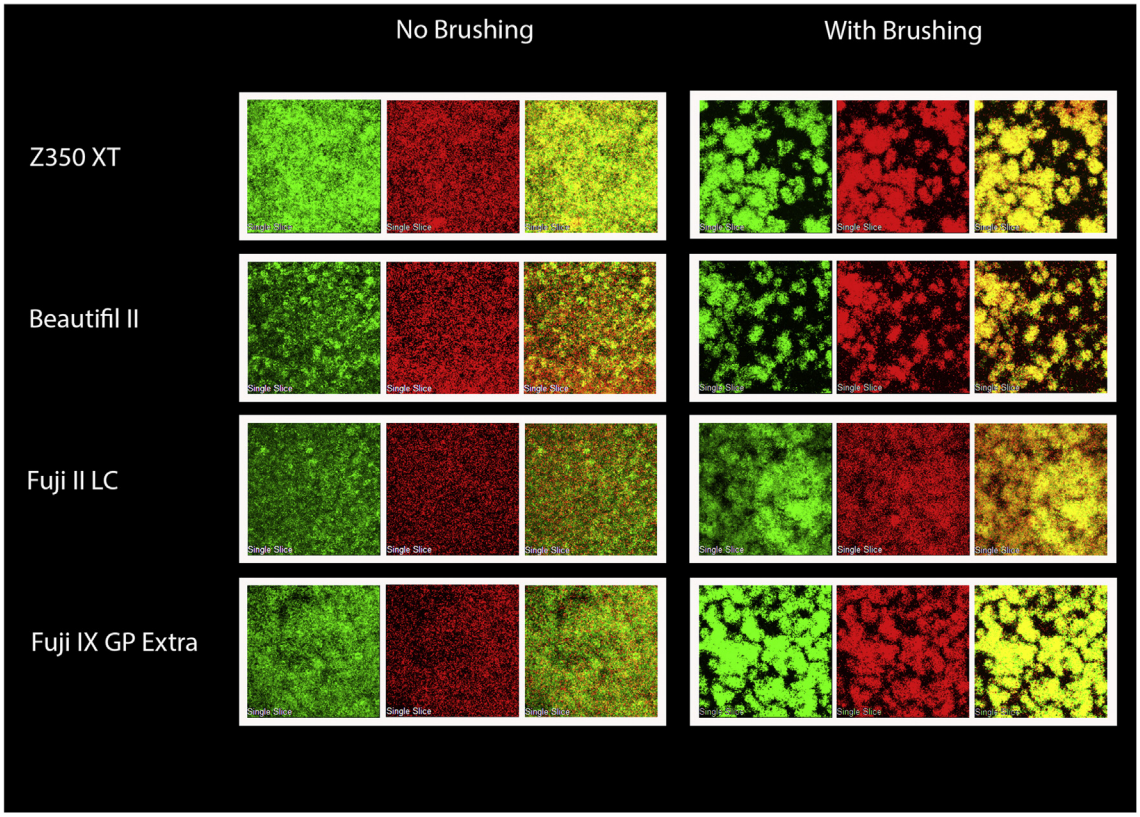

Figure 3 Representative images of *S. mutans* biovolume and live/dead cells under CLSM. The optical slices combining all Z-stack slices are shown. Live and dead cells are shown in green and red, respectively. The combination between live and dead cells is represented in yellow.

to single-paste systems such as resin-based material, Z350XT and Beautiful II.[27] The mentioned roughness was affected by physical and mechanical factors. Clinically, there were other factors that could influence the surface roughness of materials, such as chemical and biological factors. This study only interested in the material mechanical ageing by brushing, further study should be conducted to investigate the chemical and biological effect on surface roughness of materials.

The second part of this study was to evaluate the amount of fluoride released from each material. Fluoride is commonly used as a preventive treatment for dental caries and is incorporated into many restorative materials to stimulate the remineralization of tooth structure and to inhibit demineralization.[28,29] Fluoride is also known to have an antibacterial effect by interfering with bacterial metabolism.[30,31] Fluoride-releasing materials include glass-ionomer materials, resin-modified glass-ionomer materials, compomers, giomers and glass-filled resin-based materials. However, their antibacterial effects are still controversy. In this study, glass-ionomer-based materials demonstrated the highest amount of fluoride released under brushing and no brushing conditions, followed by Beautifil II and Z350XT, respectively, which was consistent with the study of Bansal and coworkers.[7] The amount of fluoride released from glass-ionomer materials, resin-modified glass-ionomer materials and giomers had an initial burst effect that decreases gradually. They also had a fluoride recharging effect.[7] However, the recharging process depends on recharging agents and their frequency of exposure. One of the most common fluoride-recharging process are the brushing with fluoridated toothpaste.[4] Although tooth brushing is the most effective way in removing biofilm, it is also one of the material mechanical ageing procedure. The 20,000 strokes brushing simulation with toothpaste in this study mimicked 2 years of daily brushing.[24] The study by Hani M. Nassar showed that the amount of fluoride released from glass ionomer-based materials were decreased after undergone brushing since high fluoride release only in early period and abated significantly to reach an ambient sustained fluoride release over time.[32] Supported by the study of Wiegand A. which stated that brushing leads to a reduction of the surface concentration of fluoride.[33] Consequently, the amount of fluoride released significantly decreased after the brushing simulation in all materials except Z350XT. As the resin composite has no fluoride releasing effect, the amount of fluoride released remained unchanged. On the other hand, glass ionomer-based materials still showed the highest amount of fluoride release. Nevertheless, the amount of fluoride released could be affected by several experimental procedures, such as the rinsing process during specimen preparation and the incubation of specimens in filtrated saliva and even in *S. mutans* culture media. There was also a time delay between the preparation of the specimen and when the measurement of fluoride release was performed.

Hence, the initial burst level of fluoride could not be measured.

The last part of this study was on *S. mutans* biofilm formation on different materials. Biofilm formation in the oral cavity together with a loss of oral microflora homeostasis is a starting point for many oral diseases, such as dental caries and periodontal disease.[34] This study tested biofilm formation on available restorative materials that have different antibacterial compositions. Both Fuji II LC and Fuji IX GP Extra have a fluoride releasing effect from their fluoroaluminosilicate glass particles, while the fluoride and boron releasing effect of Beautiful II is from the S-PRG filler.[35]

Biovolume, or the bacterial densities, is used as a quantitative parameter in addition to biofilm structures provided by the use of three-dimensional CLSM. The actual CLSM image was a multiple 2-dimensional slices assembled to form a 3-dimensional reconstruction, in Fig. 3 it was an overlay of all slices. In this study, the calculation of the bacterial density used a software to count all the fluorescently labeled voxels over a study area in all slices. From Fig. 3, an overlay image of all slices, the bacteria in brushing group seems to be packed densely together compared to the widely dispersed in no brushing group. However, in Fig. 2 which are the cross section of the same 3-dimensional reconstruction from Fig. 3 showed a denser and thicker biofilm in brushing group which coincide with the quantitative data in Table 4. Although, a positive correlation between surface roughness and biofilm formation on resin composite with no bioactivity property has been reported.[36] The surface roughness of resin composite group in this study were not significantly difference with and without brushing, no difference was found in the biofilm formation too. On the other hand, the surface roughness of no brushing Z350 XT was not significantly difference with the no brushing Beautifil II, but there was a significant difference in their biovolume. In this study, we were using materials with different degree of bioactivity, the biofilm formation could be influenced by more than just surface roughness. As mentioned before, some other ions released from the Beautifil II, Fuji II LC and Fuji IX GP Extra could have the influenced on the biovolume. According to a previous study on the biofilm sensitivity of different *S. mutans* strains to various fluoride levels, *S. mutans* ATCC 25175 was found to be more sensitive to a higher concentration of fluoride, and less biofilm mass was produced together with less extracellular polysaccharides (EPS).[37] No known antibacterial composition of resin composite together with a monomer composition, triethylene glycol dimethacrylate (TEGDMA), had growth-stimulating effects on caries-associated microbes.[38] This might affect the higher biovolume on the surface of resin composites. On the other hand, in the material that underwent brushing simulation, the biovolumes were not significantly different among all the materials. When comparing within each material, the brushing simulation resulted in a statistically higher biovolume in Beautifil II, Fuji II LC and Fuji IX GP Extra as a result of the gradual decrease in fluoride ions after brushing simulation.[7] Meanwhile, Z350XT was the only material in this study that showed no difference in the biovolume of *S. mutans* in both the with and without brushing simulation groups due to its lack of antibacterial effect.

As shown in this study, the different materials and surface abrasions affected the surface roughness. Because of surface irregularities, bacteria can attach more frequently and survive longer. Irregularities can protect bacteria from natural removal forces and oral hygiene measures.[39] A surface roughness of 0.2 μm is the minimum roughness that easily promotes the adherence of microbes.[40] In this study, only the resin-based materials had a Sa value less than 0.2 μm under both brushing conditions. However, the biovolumes of all the materials in this study did not correspond to the Sa values. The glass-ionomer-based materials with Sa values greater than 0.2 μm had no significant differences in biovolume when compared to that of the resin-based materials. Results from this study is contrary to the study of Flausino and coworkers, who reported that there was a positive correlation between the biovolume and average absolute roughness of resin composites, conventional glass-ionomer materials and resin-modified glass-ionomer materials.[41] There might be several factors in this study contributing to different degree of biofilm formation. However, the surface irregularities should be clinically considered.

Another piece of information obtained from this study is the viability or the live/dead ratio of *S. mutans* in the biofilms. The number of live and dead cells within bacterial biofilms has been commonly used as a quantitative parameter in recent studies.[42–44] Glass-ionomer-based materials and S-PRG-filled resin composites (giomers) are known to have antibacterial properties. Therefore, this study attempted to assess the antibacterial properties of materials on biofilms formed on restorative materials. The biofilm was stained with SYTO9 and propidium iodide dyes to differentiate between the green color of live cells and the red color of dead or damaged cells in the biofilm.[45] The results showed no difference in live/dead bacteria either within or between material groups, even though the average absolute roughness and fluoride ion release were significantly different. This was contrary to the study of Nedeljkovic and coworkers, which demonstrated a strong inhibitory effect of glass-ionomer materials and giomers on the growth of *S. mutans*.[6] In addition, the study of Aykent and coworkers proposed that there was a positive correlation between the viability of *S. mutans* and surface roughness.[13]

According to the study by Yoshihara and coworkers comparing *S. mutans* inhibition properties of conventional resin composite, S-PRG filled resin, glass-filled resin and conventional glass-ionomer materials, they found that all materials failed to inhibit the growth of *S. mutans*.[35] In correspond with this study, even though fluoride was released from Beautiful II, Fuji II LC and Fuji IX GP Extra, the concentration of fluoride released was not enough to exhibit antibacterial properties. A minimum concentration of fluoride ions at 250 ppm is needed to inhibit bacterial growth.[35] This study determined that the maximum level of fluoride ions was 19.4295 ppm in the no brushing simulation of the Fuji IX GP Extra group. We suggested that a low amount of fluoride ions was released and detected from fluoride-releasing materials; therefore, the effect on bacterial inhibition may have been low. Moreover, after brushing, the biovolume on Fuji IX GP Extra were significantly increased, while the live/dead ratios of *S. mutans*

were in decreasing tendency. It could be possible that there were more dead *S. mutans* in a denser biofilm which leads to a slightly decreasing of their live/dead ratios.

Fluoride-releasing dental materials are likely recommended for high caries risk patients to prevent further development of carious lesions. However, within a limitation of this study, fluoride release from those materials was insufficient to prevent the colonization of the caries-related bacterium *S. mutans*. Routine oral cleansing is still highly essential, as this study showed no significant difference in *S. mutans* biovolume and live/dead on different materials after undergoing brushing in a short period of time. Furthermore, there are many other aspects of the effect of fluoride-releasing materials to be studied, and a long-term study may be more beneficial to determine the effectiveness of these fluoride-releasing materials.

All the tested materials demonstrated good surface stability within the time limitation of the testing. The Sa was material-dependent. The amount of fluoride released was material- and time-dependent, except for Z350XT. Brushing simulation had a negative effect on *S. mutans* biovolume on Beautifil II, Fuji II LC and Fuji IX GP Extra. Z350XT, Beautifil II, Fuji II LC and Fuji IX GP Extra had the same biovolume after undergoing aging by brushing simulation. The brushing simulation and different material types had no effect on the live/dead cell ratio of *S. mutans*. Further study should be performed in a longer period of time to achieve the relationship between time and bacterial viability.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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