Template assisted surface micro microstructuring of flowable dental composites and its effect on the microbial adhesion properties

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Abstract

Objectives: Despite their various advantages, such as good aesthetic properties, absence of mercury and adhesive bonding to teeth, modern dental composites still have some drawbacks, e.g., a relatively high rate of secondary caries on teeth filled with composite materials. Recent research suggests that micro structured biomaterials surfaces may reduce microbial adhesion to materials due to unfavorable physical material-microbe interactions. The objectives of this study were, therefore, to test the hypotheses that (i) different surface micro structures can be created on composites by a novel straightforward approach potentially suitable for clinical application, and (ii) that these surface structures have a statistically significant effect on microbial adhesion properties.

Methods: Six different dental composites were initially tested for their suitability for micro structuring by polydimethylsiloxane (PDMS) stamps. The composites were light-cured between glass slides and micro-structured PDMS stamps. The nano-hybrid composite Grandio Flow was the only of the tested composite with satisfying structurability, and, was therefore used for the bacterial adhesion tests. Composites samples were structured with four different micro structures (flat, cubes, linear trapezoid structures, flat pyramids) and incubated for 4 hours into centrifuged saliva. The bacterial adherence was then characterized by colony forming units (CFU) and scanning electron microscopy (SEM).

Results: All four micro structures were successfully transferred from the PDMS stamps to the composite Grandio Flow. The CFU-test as well as the quantitative analysis of the SEM images showed the lowest bacterial adhesion on the composite samples with the smooth surfaces. The highest bacterial adhesion was observed on the composite samples with linear trapezoid structures, followed by flat pyramids and

cubes. The microstructure of dental composite surfaces statistically significantly influenced the adhesion of oral bacteria.

Significance: Modifying the composite surface structure may be a clinically suitable approach to control the microbial adhesion and thus, to reduce the risk of secondary caries at dental composite restorations. Smaller composite surface structures may be useful for accomplishing this.

Keywords:

Dental composites, micro structuring, bacterial adhesion

Introduction

Since the introduction of resin-based dental materials near the middle of the last century, composite restorations in dentistry became indispensable because of the patients aesthetic demands and ease of composite processing [1]. Composites are a mixture of organic and/or inorganic fillers surrounded by a monomer matrix which can be set-on-command by photopolymerization e. g. with blue LED lamps [2]. Depending on their filler particles sizes composites can for example be categorized into four different groups: macro-, micro-, hybrid- and nanofiller composites. In addition, they can be categorized according to their rheological properties into flowable and non-flowable composites.

Despite their advantages, such as good aesthetic properties, absence of mercury and adhesive bonding to teeth, dental composite still have some drawbacks such as for example the polymerization shrinkage, their tendency to absorb water [3] and the onset secondary caries caused by microbes in teeth filled with composite materials [4].

A challenge arises through the surface treatment of composites. Inappropriate finishing procedures may result in increased surface roughness [**Error! Bookmark not defined.**~~7~~,**Error! Bookmark not defined.**~~8~~,5]. An important property in relation to the structural surfaces roughness of dental composites is the adhesion of oral microbes to the composites. Surface roughness influences bacterial colonization [6], particularly on composite materials [5~~11~~]. Smooth surfaces are preferred clinically, because of their relatively low bacteria adherence [7]. Carlén *et al.* reported, however, that a polished hybrid composite to accumulate more bacteria than the unpolished one [5~~11~~].

Some investigations showed that microbes adhere stronger on composites surfaces than to the natural tooth covered by a pellicle [8] or in comparison to other dental materials. A threshold level of composite surface roughness of R_a = 0.2 µm has been discussed, below which no further reduction in microbial accumulation could be expected [9], however, no convincing explanation for this has been given. Although surface roughness seems to be an important factor for microbial accumulation on dental composites, materials properties such as filler-size [9~~15~~] shape and content [10], composite surface tension [11], chemical surface composition [12], protein adsorption [5~~11~~] and others seem to be important factors as well.

It has been predicted that future commercial dental composites will possess antimicrobial properties [13] and the number of scientific literature addressing this subject has grown strongly in the last years [14]. Approaches to equip resin based dental materials with antimicrobial properties include silver [15] or zinc oxide nano particles [16], silver-supported antibacterial materials [17], zinc oxide eugenol [18], quaternary ammonium functionalities [19,20], alkylated ammonium chloride derivatives [21], chlorhexidine diacetate (CHXA) [22], carolacton [23] and others. The addition of antimicrobial agents to composites, however, may lead to reduced mechanical properties of the composites and in many cases the antimicrobial effect of the composites is not maintained [13~~19~~].

A new and promising approach to reduce the microbial adhesion to different biomaterials surfaces uses specific micro or nano surface topographies or patterns [24,25,26,27,28]. The microbial adhesion reduction mechanisms of these materials surfaces are still an enigma, but some authors assume that an unfavorable physical interaction between microbes and the materials surface is responsible for their antimicrobial effect [27~~34~~,28~~35~~].

Reducing microbial adhesion to materials with this approach has a number of advantages since it uses neither antibiotics nor other chemical antimicrobial agents or compounds. Hence these materials cannot lead to antibiotic resistance of microbes or negative side effects of drug release such as cytotoxicity to body cells.

Based on these findings, the question arises if a surface structuring approach is also feasible for reducing microbial adhesion to dental materials such as dental composites. Little is known about the interaction of microbes and flowable composites. Due to their rheological properties flowables seem to be, however, the ideal materials for surface patterning.

It was, therefore, the aim of this current study to test the hypotheses that (i) different surface micro structures can be created on composites by a novel straightforward approach potentially suitable for clinical application and (ii) that these surface structures have a statistically significant effect on microbial adhesion properties when compared with flat control samples of the same composite. To the best of knowledge, both have not been attempted previously and may, if successful, lay the foundations to a new way of functional surface structuring of dental composites.

Materials and methods

Dental composites

Six different composites were first tested for their structurability by polydimethylsiloxane (PDMS) stamps: The nanohybride composites CLEARFIL MAJESTY Posterior (CMP; Kuraray Europe GmbH, Frankfurt, Germany), Grandio Flow (GF; Voco GmbH, Cuxhaven, Germany); Premise (P; Kerr Corporation, Orange, USA), Tetric EvoFlow (TEF; Ivoclar Vivadent AG, Schaan, Liechtenstein), Venus Diamond (VD; Heraeus Kulzer GmbH, Hanau, Germany) and the microhybride composite XFlow (XF; Dentsply International, York, USA). All composites used in the current study were of the shade A2. The properties of the composites, as obtained from manufacturers data sheets, are listed in Tab. 1.

PDMS-stamp assisted micro structuring

Cut pieces of silicon (Si) wafers, with a size of 10 mm × 10 mm, and a thickness of 0.5 mm with three different surface structures created by photolithography (cubes, linear trapezoid structures, flat pyramids) and flat native Si as control were used as initial master pattern samples. The exact dimensions of the structures can be found in the supplementary part. Initially only the cube structures were used for testing the structurability of the different composites.

The structures were transferred from the Si masters to polydimethylsiloxane (PDMS) stamps made of PDMS Sylgard 184 (Dow Corning Corporation, Midland, USA). The ratio between pre-polymer and curing agent was 10:1 wt./wt. This liquid was poured on the Si masters and subsequently cured for 24 h at 75 °C resulting in a flexible PDMS stamp. Flat PDMS stamps cured on flat, unstructured Si were used for the creation of flat control samples.

155 Approximately 0.1 ml of the flowable composite material was deposited on a glass
156 slide. Subsequently, the composite was covered with the structured PDMS stamp
157 (size 1 cm × 1 cm), and a slight pressure was applied to the stamp by hand.
158 Afterwards, the composite was polymerized for 30 sec by an Elipar FreeLight 2 LED
159 light curing unit (LCU; 3M ESPE AG, Seefeld, Germany) by subsequently curing
160 overlapping areas covered by the light guide of the LCU. This procedure was applied
161 to different composites. The thickness of the so structured and cured composite
162 samples was approximately 1 mm. The different steps of the PDMS-stamp assisted
163 micro structuring of the dental composites are illustrated schematically in Fig. 1.
164 For microbial adhesion tests in the well plates, the composite samples were
165 structured from both sides to avoid an influence of the un-structured bottom side of
166 the samples on microbial adhesion results.
167 The surface structure of the micro structured composites was characterized using a
168 Zeiss Auriga 60 scanning electron microscope (SEM; Zeiss AG, Oberkochen,
169 Germany). The pattern reproduction at the composites surfaces was qualitatively
170 judged by eye from the SEM micrographs.
171
172 *Rheological characterization of dental composites*
173 The Advanced Rheometric Expansion System (ARES, TA Instruments Inc., New
174 Castle, USA) was used for the rheological characterization of the composites in this
175 study. The temperature during all measurements was 25°C. The flowable resin
176 composites were squeezed on the lower part of a parallel plates viscometer module
177 (diameter 25 mm). The gap between the two plates was fixed to 1 mm. Strain sweep
178 measurements were performed on each material to determine the maximum strain,
179 at which the resin still behave like a structured fluid (solid-like). A strain value lower
180 then this maximum strain was chosen individually for each composite. Therefore, the

complex viscosity (η*) of the different composites achieved from frequency sweeps (0.1 rad/s, 1 rad/s, 10 rad/s and 100 rad/s) are comparable.

Additionally, stress relaxation tests were used to investigate the flow behavior of the resin composites after instantaneous shear strain. To mimic the conditions during the preparation of the samples a pre-shear rate of 10 s^{-1} was used for 5 s before applying a strain of 10 %. The resulting shear modulus was measured as a function of time.

Contact angle measurements on the dental composites

It is well known from literature [27~~34~~,29] that the bacterial adhesion to a surface is affected by its wettability. Therefore, water contact angle measurements were carried out to determine the wettability of the micro-structured composites and the un-structured control samples. The static contact angle was determined with the sessile drop method using a Drop Shape Analysis System DSA 10 (Krüss GmbH, Hamburg, Germany). For statistical analysis, ten measurements with deionized water were carried out on respectively 3 samples and averaged.

Microbial adhesion and biofilm formation test

Microbial adhesion and biofilm formation as function of the dental composites surface micro structures was investigated using oral microorganisms originated from a test person (age 25) with a DMF-T-index (decayed-missed-filled-teeth) of 0. Plaque was sampled from the test person from each quadrant using sterile paper tips and incubated anaerobically in Schaedler nutrient solution (OXOID Deutschland GmbH, Wesel, Germany) for 24 h to induce microbial growth. Afterwards, the microbial suspension was adjusted with fresh nutrient solution to an optical density (OD570nm) of 0.5 using a UV-VIS photospectrometer (Eppendorf PCP 6121, Eppendorf AG, Hamburg, Germany). Simultaneously, the composite specimens were, first, incubated in deionized water for 7d at 37 °C to remove free radicals left from the

polymerization reaction. Afterwards, for pre-conditioning of the micro structured composite surfaces, saliva was collected from the test person after tooth brushing in the morning, centrifuged (Eppendorf 5415D, Eppendorf AG) for 5 min at 13000 rpm (16110 g) and the composite specimens were incubated for 1 h at 37 °C each in 500 µL of the supernatant. The pre-conditioned composite samples were rinsed twice in phosphate buffered saline (PBS) and incubated for 4 h with the microbial suspension while gently mixing using a plate shaker (Titramax 100m, Heidolph North America, USA) at 150 rpm to reduce the effect of sedimentation of the microbial cells on adhesion.

To investigate the specific influence of the composite surface micro structures on bacterial adhesion and biofilm formation and to exclude an influence of specific chemical surface properties of the different dental composite materials, adhesion and biofilm tests were performed only on the specimens prepared from the GF composite.

Biofilm analysis

The established biofilms were quantitatively characterized by estimation of the colony forming units (CFU) and scanning electron microscopy. Before analysis, all samples were carefully rinsed with PBS, to remove non-adherent microorganisms.

For estimating the CFU numbers, composite specimens were placed each in 1 mL of PBS and microorganisms adherent on the samples` surfaces were removed using a vortexer. 20 µL of each microbial suspension was, as the first step, diluted 1:10 in physiological saline solution. Afterwards, a dilution series was prepared with the highest dilution of 10^{-6}. Each dilution step was incubated under anaerobic conditions for 48 h at 37 °C on agar plates prepared with Schaedler nutrient solution. The CFU were estimated by counting.

For SEM, composite samples with adherent microorganisms were incubated in glutardialdehyde solution (2 %) for 30 min at room temperature followed by an incubation step with cacodylate buffer (0.1 M) for 10 min. After fixation, samples were washed for 10 min in PBS buffer solution and twice in deionized water. Samples were, then, dehydrated using an ascending ethanol series from 30 % to 96 %. Dehydrated samples were air dried for 24 h and sputter coated with gold (S150B, Edwards Ltd, Crawley, UK). For imaging, the AURIGA 60 SEM was operated at 3.5 kV and a working distance of about 3 mm.

Next to CFU counting, the numbers of adherent microbial cells were additionally estimated based on the SEM images by direct counting, as well.

Statistical analysis

The data were analyzed for statistically significant differences among groups using a one-way ANOVA (analysis of variance) based on a Tukey post-hoc comparison (Sigmaplot 12.0, Systat Software Inc., Chicago, USA). The level of significance was $p \leq 0.05$. All data are given as means ± standard deviations.

Results

The SEM micrographs (Fig 2a-f) of the initial stamp assisted surface micro structuring experiment of the composites presented in Tab. 1 reveal the successful pattern creation for GF (Fig. 2b) with a good surface pattern definition of the cubes. Although a cube surface structure was also partially accomplished with the CMP composite (Fig. 2a), the surface pattern definition is clearly poorer compared to the GF surfaces. In some surface areas of the CMP samples the structures (cubes) are completely absent (e. g. the upper right corner of Fig. 2a), in others they are poorly reproduced (e. g. the center of Fig. 2a).

The surfaces of the other four composites (Fig. 2c-f) show no cubic surface patterns, but rather rough surfaces which are dominated to different extends by the filler particles. This is most clearly visible for the VD composite which surface shows the filler particles most distinctively (Fig. 2e).

The complex viscosity (η*) of the different composites shown in Table 1 obtained from frequency sweeps (0.1 rad/s, 1 rad/s, 10 rad/s and 100 rad/s) increased in the following order: XF, GF, P, VD, TEF (Fig. 3a). For all composites, the complex viscosity decreases with increasing frequency.

The stress relaxation was the fastest for the composite GF, followed by XF, VD, P and TEF (Fig. 3b). Due to a low interfacial adhesion between the dental composite CMP and the plates of the rheometer and the composite’s consistency and texture, no strain could be transferred to the composite. Therefore, no data could be obtained about the rheometric behavior of this dental composite.

Since GF was the only composite with a sufficient surface structurability, this composite was used for further patterning tests and microbial adhesion tests.

Fig. 4 shows the flat, respectively, patterned Si masters (Fig. 4a,c,e,g) compared to the structured GF composite surfaces (Fig. 4b,d,f,h) using the respective Si masters.

For preparation of the control samples, a flat Si master (control master) (Fig. 4a) was used. While the composite cured against the flat Si maters is completely flat (Fig. 4b), the surface structures (patterns) cubes (Fig. 4d), linear trapezoid structures (Fig. 4e) and flat pyramids (Fig. 4h) have been reproduced well on the GF surfaces. All faces of the original Si maters, i. e. flat ones, the cubes, faces of the linear trapezoid structures and flat pyramids, respectively, have been reproduced with a good pattern quality at the GF composite surfaces.

On each of the three different structured surfaces of the composite, the composites' filler particles are clearly visible (Fig. 4d,f,h). On the composite surface structured with cubes (Fig. 4d), the filler particle are most dominant but are also clearly present on the other two structured composite surfaces (Fig. 4f,h). The filler particles to some extent disturb the qualitative pattern fidelity at the composite surface as can be seen for example by individual filler particles protruding from the side faces of the linear trapezoid structures in Fig. 4f.

The water contact angles of the GF surfaces increased after structuring (Fig. 5). The water contact angels were 80.3° ± 2.6° on the flat composite surfaces (control), 99.7° ± 2.6° on the cube patterned surfaces, 101.8° ± 4.3° on the linear trapezoid patterned surfaces and reached its highest value on the composite surfaces patterned with the flat pyramids with 129.5° ± 2.5°.

After 4h of incubation, on the flat control samples only few microbial colonies each consisting of approximately 2 to 40 microbes have been observed (Fig. 6a). The number of these colonies and microbes within the colonies increased on the structured composites surfaces (Fig. 6b-d). A statistically significant difference in CFUs was observed between the flat control sample and the linear trapezoid patterned surfaces. The cube structured composite surfaces showed the lowest number of microbes (determined by SEM and image analysis) among the structured

composite surfaces (Fig. 6b), whereas the linear trapezoid patterned surfaces showed the highest number of microbes (statistically significantly different compared to the 3 other surfaces; Fig. 7b) and the largest size of microbial colonies (Fig. 6c). On all samples, the shape of the microbes was identified as spherical and the microbes were frequently arranged in strands. On the structured composite surfaces, several of the microbial strands were aligned along surface topographic features. Some strands are aligned parallel to rows of cubes (Fig. 6b). On the linear trapezoid patterned surfaces, the majority of the microbes were located at or near to the side walls of the linear trapezoid structures and the strands were aligned along or perpendicular to the long axes if the linear trapezoid structures (Fig. 6c). Most of the microbes that adhered on the flat pyramid structured surfaces were found at the base edges of the pyramids and the alignment of some microbial strands was parallel to the base edge direction as can be seen in Figure 6d. The filler particles prominent at the surface of the structured composites seem to not noticeably affect the microbial adhesion since there was neither an increased number nor a reduced number of microbes found at or nearby the particles.

Discussion

Structure and properties of dental composites surfaces depend on their composition and the finishing procedures (i.e. polishing) applied to the composite, which in turn may affect microbial adhesion properties. In the current study, we developed a novel approach to create different composite surface structures and affected through this microbial adhesion properties of the composites.

More microbes adhere and accumulate to dental composite than to other restorative dental materials *in vitro* [9~~15~~,30,31] and *in vivo* [32,33] due to their lack of antibacterial activity, e.g., compared to amalgam. Adjacent to the restoration margins of the dental composite, this may lead to secondary caries *in vivo* and, thus, shorten the life of composite restoration [34].

Effective antimicrobial dental composites are needed to prevent or reduce problems associated with the adhesion of microbes to dental materials surfaces including plaque accumulation i. e. biofilm formation, (secondary) caries, discoloration, gingivitis and others. Nevertheless, current commercially available or experimental dental composites do not or only partially solve these problems.

Reduction of biofilm formation on dental materials and the development of antimicrobial dental materials are timely research areas [13~~19~~]. While for the former, polishing approaches seem to be effective [10~~16~~] addition of antimicrobial particles or agents [16~~22~~,17~~23~~,18~~24~~,19~~25~~,20~~26~~,21~~27~~,22~~28~~,23~~29~~,**Error! Bookmark not defined.**~~30~~] to composites have been used in the latter.

It has been reported in recent years that biomaterials surfaces in general with specific micro or nano surface topographies including specific structures [24~~31~~,25~~32~~,26~~33~~] or nanoroughnesses [27~~34~~,28~~35~~] may reduce microbial adhesion compared to surfaces on which these features are absent. This approach has not been applied and investigated for dental composites so far and was addressed in the

345 current study, to the best of our knowledge, for the first time. Therefore, the aims of
346 this study were i) to test if microstructures may be created to dental composite
347 surfaces by a method potentially suitable for clinical application and ii) if the created
348 patterns affect the microbial adhesion on these surfaces.
349 When choosing suitable materials for our study, several factors had to be
350 considered. Stamp assisted surface micro structuring requires the material to adapt
351 well to the stamp, i.e. that it is sufficiently flowing into the fine surface structures of
352 the stamp. Therefore, flowable composites were chosen in the current study.
353 It has been shown previously, that surface structures of a size of 1 nm to a few μm
354 affect microbial adhesion on materials surfaces, depending on for example the type
355 of microbes and materials, with a general tendency of smaller structures having a
356 more pronounced reducing effect on microbial adhesion [35,36,37,38]. On the other
357 hand, as can be seen from Fig. 2a-f, an important limiting factor of stamp-assisted
358 surface structuring of composites is the maximal size of the filler particles which in
359 the case of composites used ranged from 550 nm to 30 μm (Tab. 1), with four of the
360 six composites having maximal particles sizes of ≤ 1.6 μm. A stamp feature size of
361 approximately 1 μm × 1 μm × 1 μm was, therefore, chosen for the initial experiments.
362 Two of the composites (GF and CMP) showed a general structurability (Fig. 2) with
363 GF showing the best pattern fidelity. Both have a maximum particle size of 1 μm and
364 1.5 μm, respectively. Therefore, their maximum particle sizes approximately fit to the
365 stamp pattern size and does not disrupt the reproduction of the pattern. Since CMP,
366 however, has a slightly larger maximum particles size than the pattern size, its
367 pattern reproduction was overall poorer compared to GF. The two other composites
368 with a maximum particle size of ≤ 1.6 μm TEF and XF have not shown well
369 reproduced structures at their surfaces which mean that the particles size is not the

370 only factor influencing structurability. VD and P have maximal particles sizes of 20
371 μm and 30 μm, respectively, which are well above the stamp structure size and,
372 hence, may at least partially explain their poor structurability. If the filler particles size
373 exceeds the pattern size, the stiff, high modulus ceramic particles cannot adapt to the
374 small structures.
375 In addition to the particle size of the composite discussed above, the rheological
376 properties seem of critical importance for the structurability of the dental composites.
377 XF and GF showed the lowest complex viscosity of the tested dental composites as
378 well as the fastest stress relaxation (see Fig. 3). These results agree with data from
379 other studies [39,40]. In general, composites with lower complex viscosity have a
380 better ability to flow into the structures of the PDMS stamps as well as in small
381 cavities and fissures of teeth. The stress relaxation curves characterize the
382 resistance against deformation over time. A slow stress relaxation indicates a strong
383 elasticity and, thus, a solid-like behavior. In contrast, a fast stress relaxation indicates
384 a more fluid-like behavior, favorable for structuring by the presented stamp assisted
385 method.
386 Since GF was the only composite with a sufficient surface structurability with the
387 three different patterns (Fig. 4), this composite was used for further investigations
388 and microbial adhesion tests. Interestingly, the surface of the patterned GF shows
389 some particles that are larger than 1 μm. Their typical shape and the fact that they
390 are embedded into the composite surface identifies them as composite filler particles.
391 It can be assumed that the large majority of the filler particles of this composite
392 exhibit a size up to 1 μm as stated by the manufacturer, but few larger particles
393 (outliers) are present in this composite as well. These larger particles disturb the
394 qualitative pattern fidelity slightly as they cannot adapt to the pattern in the stamp.

The water contact angle on the GF samples increased after structuring from 80.3° ± 2.6° (un-structured control sample) up to 129.5° ± 2.5° (flat pyramids; Fig. 5). Thus, the dental composite becomes more hydrophobic after structuring. It is well known from literature that microstructures play an important role on producing more hydrophobic surfaces [41,42,43]. This can be explained by the model of Cassie and Baxter [4146]. Cassie and Baxter proposed that a rough surface traps air within the microstructures. Thus, the fraction of the solid-liquid interface decreases. The more air is trapped, the larger the contact angle will be. This may explain the large difference in the contact angle between the GF samples structured with flat pyramids (129.5° ± 2.5°) and with linear trapezoid patterned surfaces (101.8° ± 4.3°). In case of the flat pyramids, the crosslines may hinder the air to be displaced by water. In contrast, this can happen easily for the linear trapezoid patterned surfaces, since this structure is more “open”, i.e. it allows the air the be displaced by water in the direction parallel to the faces of the linear trapezoid pattern.

The use of dental composite materials for restoration purposes increases the risk for the development of secondary caries, since microorganisms adhere stronger to composites compared to other dental biomaterials [511,44]. In the current study, microbial adhesion on the structured composite surfaces was investigated using human saliva. The main contributor in tooth decay is *Streptococcus mutans* [45]. SEM images of the composite samples incubated with the saliva showed coccoid cells arranged in strands which were consistent in size and shape with that of *S. mutans* (Fig. 6).

Avoiding initial adhesion, aggregation and biofilm (plaque) formation of the microorganisms or even reducing this process might be the most important approach to reduce the risk for secondary caries [46], e. g. by modifying the dental composites` surface properties. In our study we found that microbial adhesion was reduced on

composite surfaces structured with cubes (structure size approximately 1 µm) and flat pyramids (structure size approximately 10 µm) compared to composite surfaces structured with linear trapezoid structures (structure size of 20 µm; Fig. 6 and 7). In the literature, this physical effect of structure size on microbial adhesion is discussed to be due to the total contact area between the microbial cell and the material`s surface, which is significantly determined by the surface structures and correlated with the total adhesion energy [47,48]. Moreover, it is assumed that microbes adherent on surfaces with structures in the micrometer range bigger than the size of the microbial cells are protected against abrasion from shear stress [49,50]. These effects might have led overall to the highest microbial adhesion on the dental composite surfaces structured with the linear trapezoid structures.

The alignment of the microbial cells to the composites` surface structure is consistent with results reported by a study of Diaz *et al.* [2431] using a different biomaterial system. The authors found that on gold surfaces with a linear pattern the microbial cells were nearly perfectly aligned to these structures. They assumed that the microbes can in that way actively maximize their contact with the material`s surface.

In addition to the physical effect of the composite surface structures on microbial adhesion, also the wettability of surfaces can influence microbial adhesion [51,52]. As discussed above, the contact angle of the composite surfaces increased due to the surface structuring. In this current study, however, no correlation between the composites surface contact angle and microbial adhesion was found. Thus, the observed differences in microbial adhesion between the differentially structured surfaces can be attributed to the influence of the structures and not to the changed contact angle.

For adequate determination of the number of adherent cells on the composite surfaces, next to CFU counting the microbes were also counted directly based on SEM images. For CFU determination, the microbial cells are detached from the surfaces by shaking, vortexing or in the ultrasonic bath. This method is accompanied by the disadvantage that the cells in the colonies formed on the surfaces are most often not sufficient separated to each other and colonies grown on the agar plates might have been formed by more than one cell. Moreover, especially for strand-forming microbial species, as for example *Streptococcus mutans* the most dominant species in human saliva, CFU counting is only limited suitable and has, thus, to be supported by other methods e. g. image analysis as in the current study. The results of both applied methods are in good agreement with each other showing the statistically significantly highest microbial adhesion on the linear trapezoid structures. The direct counting, however, revealed more precise data showing more detailed statistical differences since it considered also the strand formation of the microbes.

As shown above, stamp assistant surface micro structuring of flowable dental composites requires only a stamp and a suitable dental composite. By applying a gentle pressure to the stamp patterns can be created in dental composites. Mylar strips are frequently used in clinical dentistry as matrix strips, contour tool and/or to control the surface roughness of composite restorations [53]. It appears to be feasible to apply patterned polymer stamps clinically in a similar way to dental composite as a Mylar strip if patterned polymer strips to be created in future are used. Based on this and the results reported above the hypotheses that (i) different surface micro structures can be created on composites by and straightforward approach potentially suitable for clinical application is accepted.

As discussed above the surface structures have a statistically significant effect on microbial adhesion properties when compared with flat control samples of the same composite. Therefore the second hypotheses we tested is accepted.

Conclusion

We introduced a straightforward and innovative approach to create different microstructures on dental composite surfaces. The surface structured composites did differ in their microbial adhesion properties from flat control surfaces, an important factor in this being the geometry of the patterns. With this we opened a new route of composite surface structuring that may lead to a new range of properties of dental composite surfaces. Factors limiting the surface structurability of dental composites have been found to be the filler particle size and rheological properties of the composite. Based on the simplicity this approach may be a basis and has potential to be used in clinical situations if further developed. Future research along this direction may use smaller composite microstructures that may lead to a strongly reduced microbial adhesion compared to conventionally treated dental composites, to which the first step has been taken through this current study.

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Figures

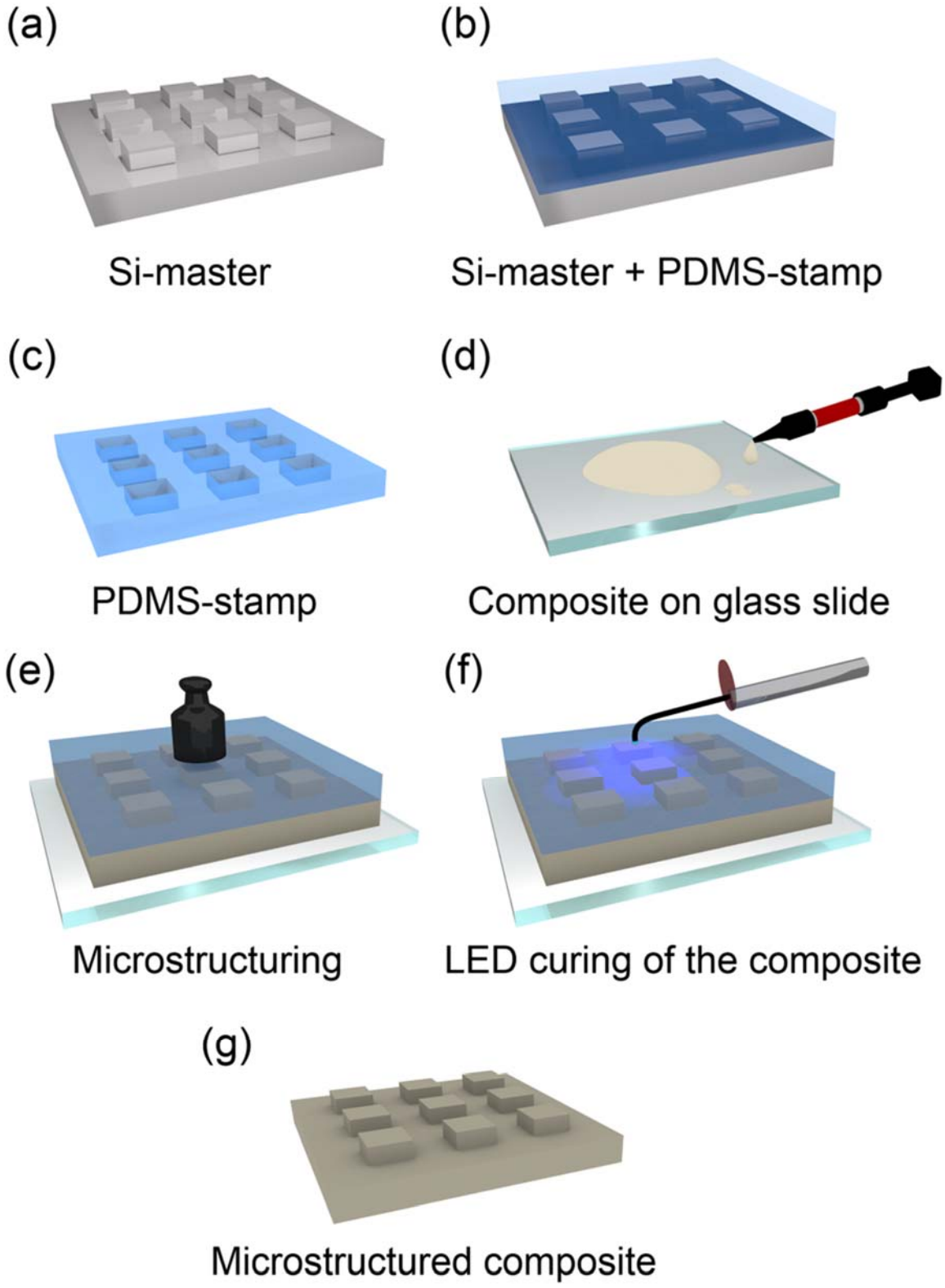


Figure 1: The different steps of the PDMS-stamp assisted micro structuring of the dental composites: (a) Initial silicon (Si) master pattern; (b) transfer of the structures to polydimethylsiloxane (PDMS) stamps; (c) PDMS stamp with negative structure; (d) deposition of the flowable composite on a glass slide; (e) covering of the flowable composite with the structured PDMS stamp, applying of a slight pressure by hand; (f)

polymerization of the composite by a light-emitting diode (LED) light curing unit for 30 s; (g) polymerized and micro-structured composite.

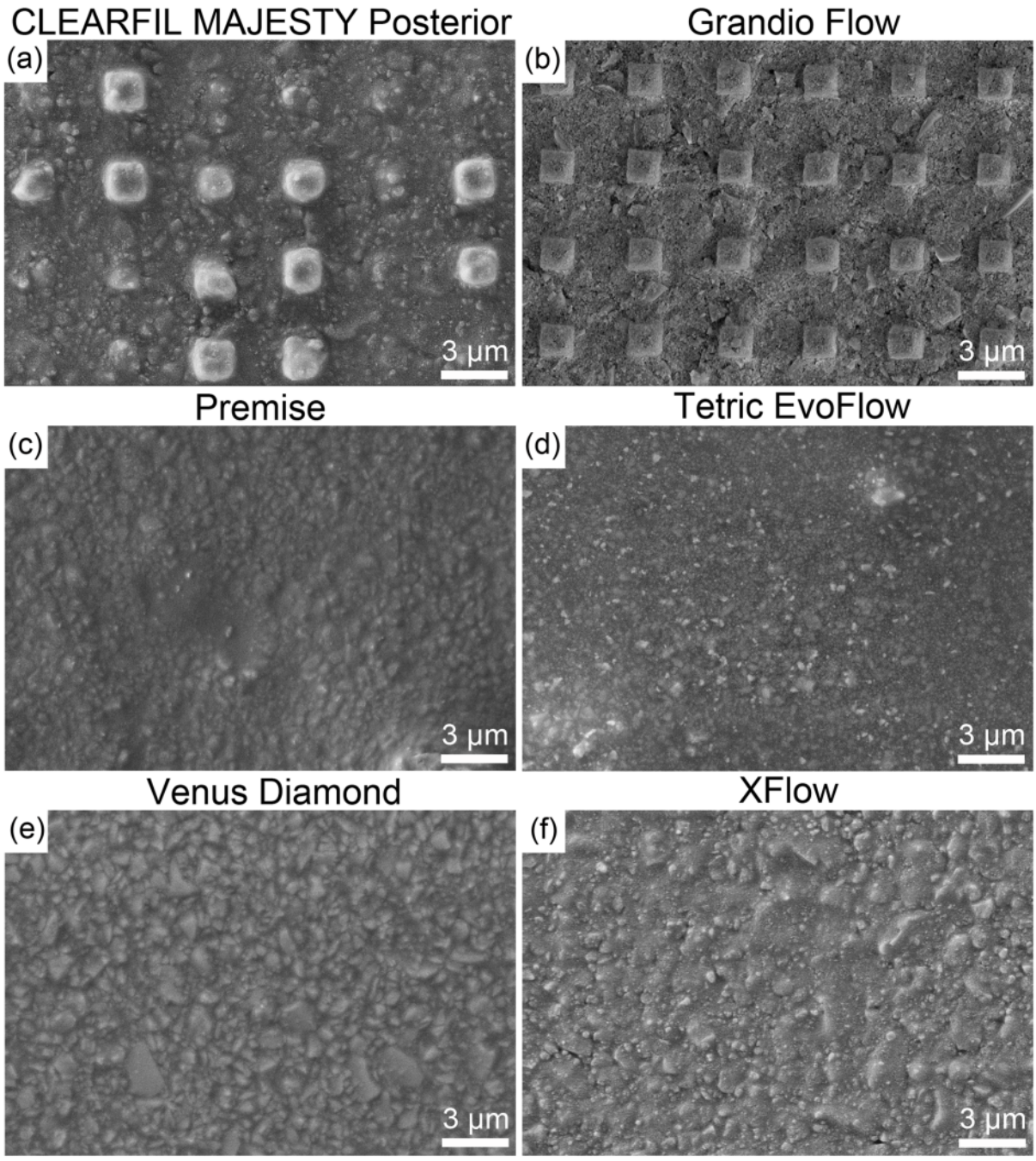


Figure 2: Results of the initial stamp assisted surface micro-structuring experiment of the different composites presented in Tab. 1: (a) CLEARFIL MAJESTY Posterior; (b) Grandio Flow; (c) Premise; (d) Tetric EvoFlow; (e) Venus Diamond; (f) XFlow.

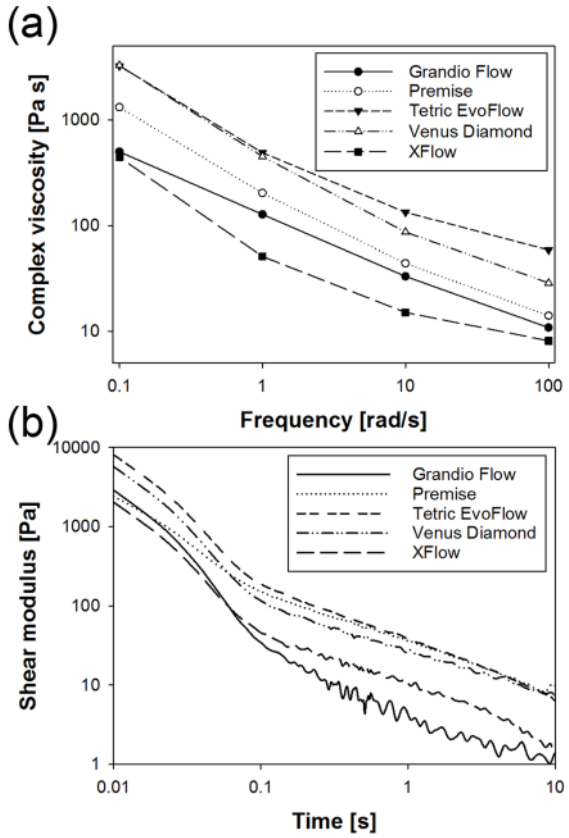


Figure 3: Rheological data of the different composites presented in Tab. 1: (a) comple viscosity; (b) stress relaxation characterized by the shear modulus as a function of time. Due to a low interfacial adhesion between the dental composite CLEARFILL MAJESTY Posterior and the plates of the rheometer no strain could be transferred to the composite, thus no data could be collected for this composite.

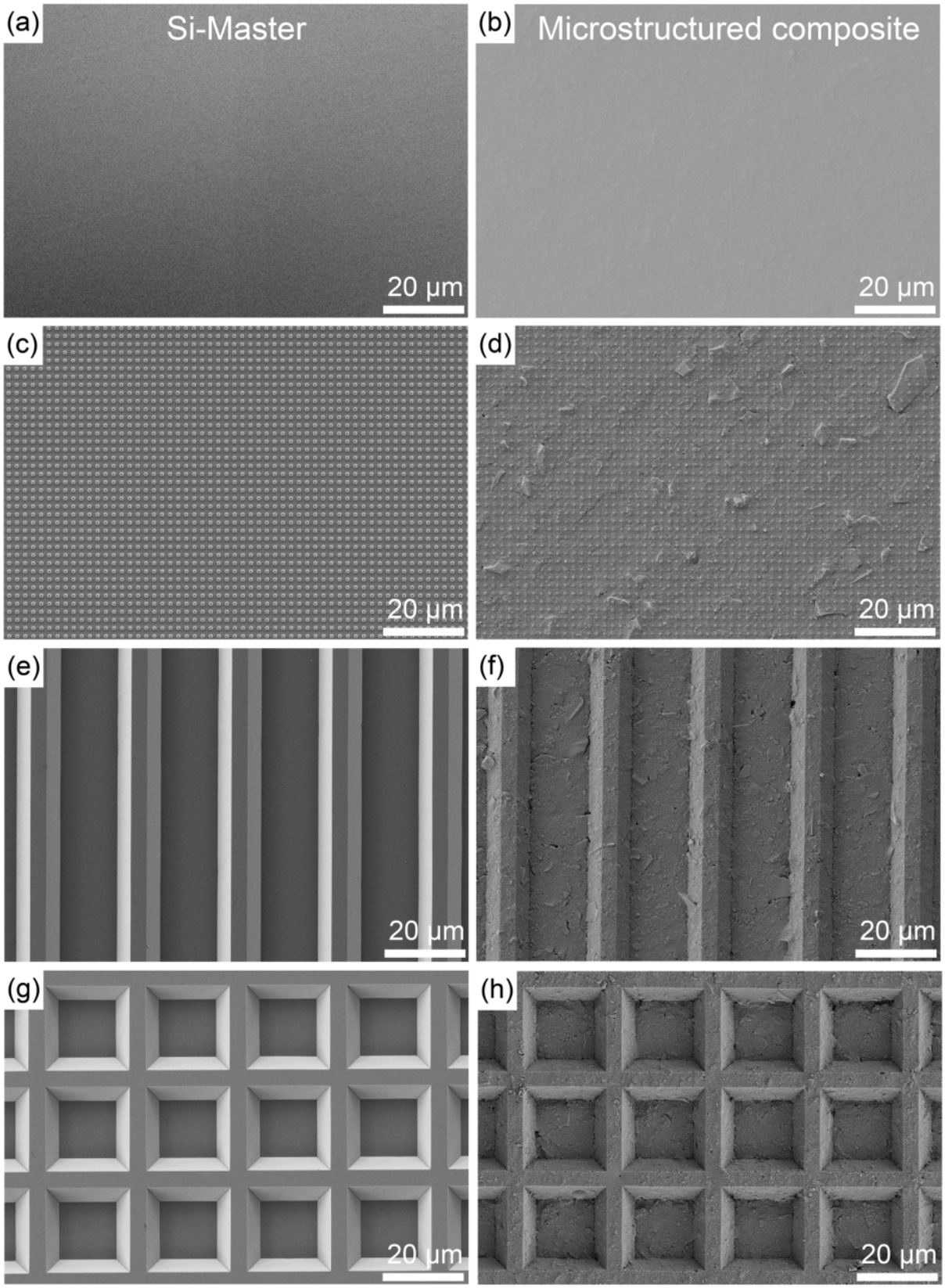


Figure 4: Comparison between the flat, respectively, patterned silicon (Si) master pattern and the structured Grandio Flow composite surface: (a), (b) flat control sample; (c), (d) cube structure; (e), (f) linear trapezoid structure; (g), (h) flat pyramids.

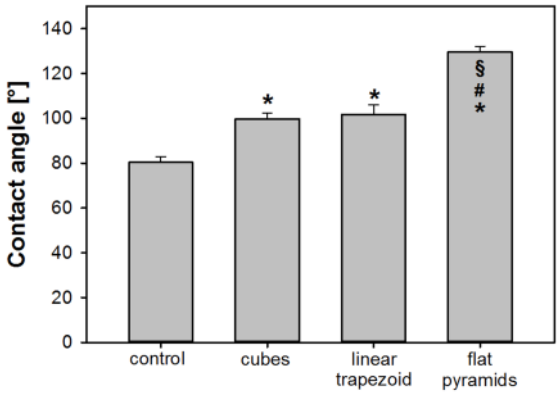


Figure 5: Water contact angle of the differently structured Grandio Flow composite surface. * p ≤ 0.05 vs. control; # p ≤ 0.05 vs. cubes; § p ≤ 0.05 vs. linear trapezoid structures.

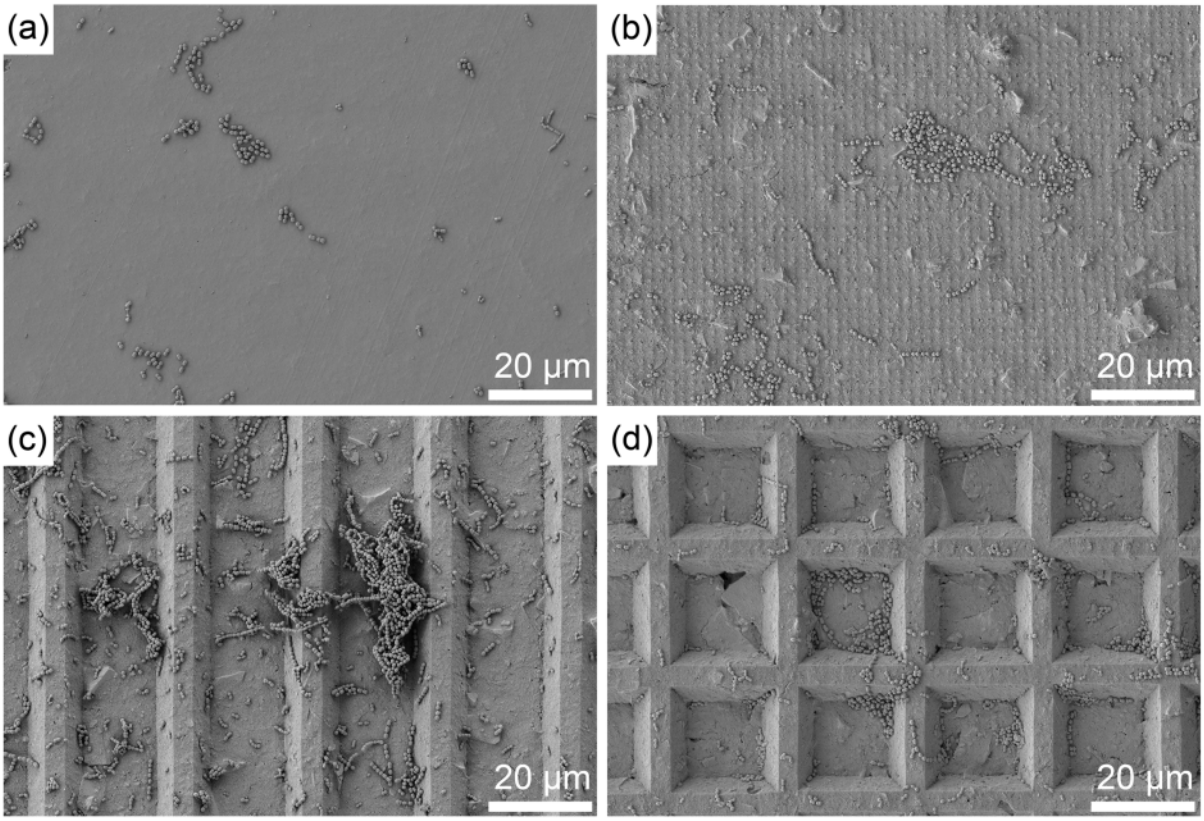
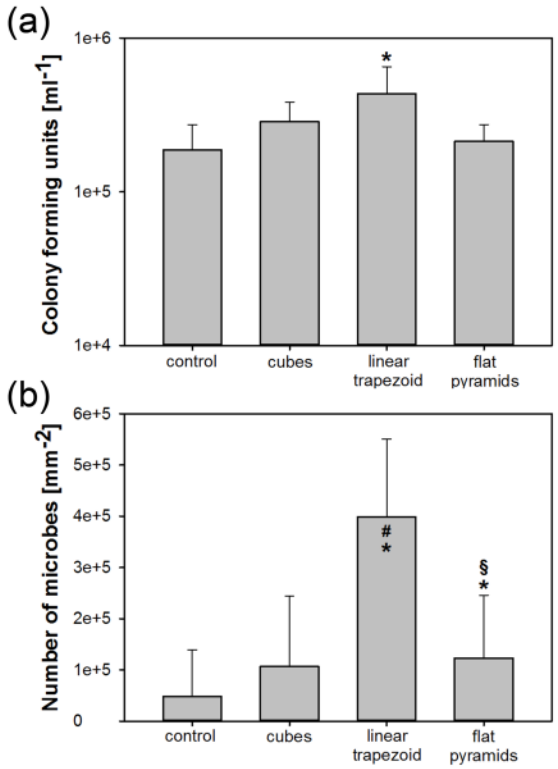


Figure 6: Microbial colonization on the flat and structured dental composite Grandio Flow: (a) flat control sample; (b) cube structure; (c) linear trapezoid structure; (d) flat pyramids.



532

533 Figure 7: Quantification of the microbial colonization on the flat and structured dental
534 composite Grandio Flow: (a) Colony forming units (CFU); (b) number of microbes
535 (determined by SEM and image analysis). * $p \leq 0.05$ vs. control; # $p \leq 0.05$ vs. cubes;
536 § $p \leq 0.05$ vs. linear trapezoid structures.

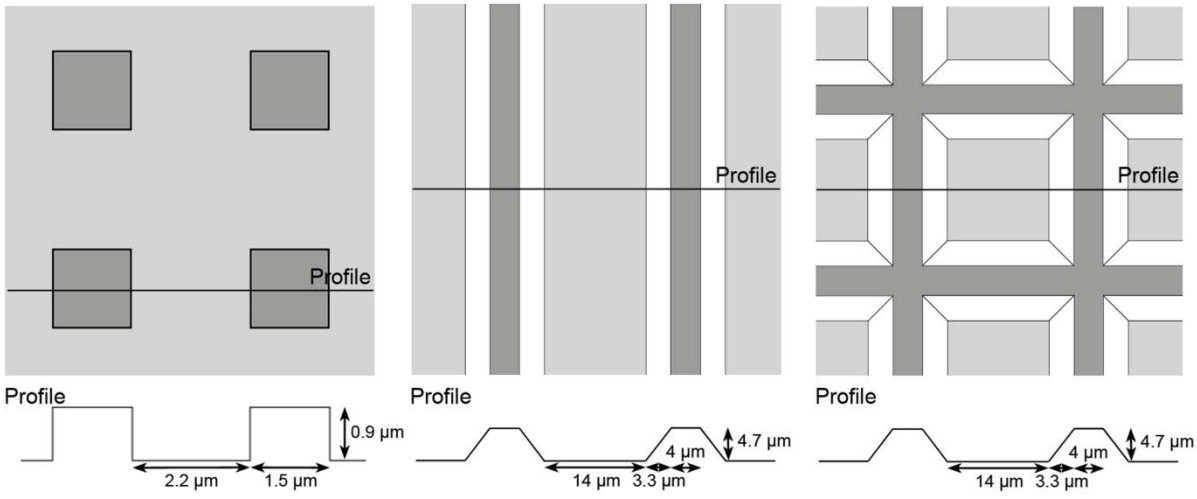
537

Tables

Table 1: Properties of the six composites tested for their structurability (data obtained from manufacturers data sheets).

Composite	Company	Filler content	Filler size	Filler material
CLEARFIL MAJESTY Posterior (CMP)	Kuraray Europe GmbH	92 % (w/w) 82 % (v/v)	20 nm – 1.5 µm	Aluminum oxide, glass
Grandio Flow (GF)	Voco GmbH	80 % (w/w) 65.6 % (v/v)	40 nm – 1 µm	Glass, SiO_2
Premise (P)	Kerr Corporation	84 % (w/w) 70 % (v/v)	20 nm/0.4 µm/30 µm	Prepolymerized fillers, glass, SiO_2
Tetric EvoFlow (TEF)	Ivoclar Vivadent AG	61.5 % (w/w)	550 nm	Glass, ytterbium trifluoride, prepolymerized fillers, mixed oxides
Venus Diamond (VD)	Heraeus Kulzer GmbH	80-82 % (w/w) 63.5-65.1 % (v/v)	5 nm – 20 µm	Glass, SiO_2
XFlow (XF)	Dentsply International	60 % (w/w)	1.6 µm	Glass, SiO_2

Supplementary data



Supplementary 1: Dimensions of the different structures on the initial silicon (Si) master pattern: left: cube structure; middle: linear trapezoid structure; right: flat pyramids.

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