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Influence of artificial ageing on surface properties and *Streptococcus mutans* adhesion to dental composite materials

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Abstract The aim of this in vitro study was to investigate the influence of artificial ageing on the surface properties and early *Streptococcus mutans* adhesion to current dental composites for the direct restoration of class II defects. Three hundred and thirty specimens each were prepared from five dental composites, and were randomly allotted to various artificial ageing protocols (storage in distilled water/ethanol/artificial saliva for 7/90/365 days; thermal cycling, 6,000 cycles 5/55°C). Prior and after each treatment, surface roughness (R_a) and hydrophobicity were determined, and *S. mutans* adhesion (ATCC 25175; 2.5 h, 37°C) was simulated with and without prior exposition to human whole saliva (2 h, 37°C). Adherence of *S. mutans* was determined fluorometrically. Means and standard deviations were calculated, and analyzed using three-way ANOVA and post-hoc analysis ($\alpha = 0.05$). For both R_a and *S. mutans* adherence to uncoated and saliva-coated specimens, significant influences of the composite material, the ageing medium and the ageing duration have been observed; for surface hydrophobicity, significant influences of the composite material and the ageing duration were found. For uncoated specimens, significant increases in *S. mutans* adhesion were observed with prolonged artificial ageing, whereas significant decreases in *S. mutans* adhesion were found for the saliva-coated specimens. The data indicate influences of the artificial ageing method on surface parameters such as R_a and hydrophobicity as well as microbial adhesion. The results underline the relevance of saliva coating on the outcome of studies simulating microbial adhesion, and highlight differences in the susceptibility of dental composites for the adhesion of oral bacteria.

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1 Introduction

In the last few years, dental composite materials gained steadily increasing importance, and their range of application is still broadening continuously. As sensitivity to moisture contamination [1], polymerization shrinkage [2, 3], limited wear resistance [4] or biocompatibility [5] are still major concerns, several improved composite materials have been introduced in the recent years, promising significant advancements with regard to these aspects. As a result, a broad number of different composite materials is available particularly for the restoration of dental class II defects, comprising particularly classical composite materials, silorane-based composites, and ormocers.

Classical dental composite materials consist of a methacrylate-based resin matrix supplemented with inorganic macro-, micro- or hybrid-filler particles, and polymerize by methacrylate functionality. For reducing polymerization shrinkage and improving mechanical properties, nano-filler particles have been introduced in the last years, which allow for a significant increase in filler volume. For further reduction of polymerization shrinkage, a special group of dental composite materials has been developed under the name *siloranes*, which derives from their major chemical building blocks *siloxanes* and *oxiranes* [6, 7]. These materials polymerize by cationic ring-opening polymerization, which partially compensates volume shrinkage during polymerization [8]. The so-called *ormocers*, which is an acronym derived from the term *organically modified ceramics*, have been introduced as another major innovation in the recent years. These materials feature a SiO_2-based

anorganic backbone, which is functionalized by incorporation of multifunctional urethane- and thioether(meth)-acrylate alkoxysilanes, and is supplemented with inorganic and organic filler particles similar to conventional composites. In contrast to conventional methacrylate-based resins, ormocers allow modification of mechanical parameters over a wide range [9].

For any dental material, long-term survival under oral conditions is of great concern; for evaluating the ageing behaviour of dental materials, the clinical ageing process is most commonly simulated in vitro. Numerous in vitro studies have been focussing on the influence of artificial ageing on the mechanical performance of dental composite materials by means of thermal cycling and mechanical loading (TCML), or storage in aggressive liquids [10–15]; however, surprisingly little evidence is available on the adhesion of oral microorganisms to the surfaces of artificially aged composite materials. It is a well-known fact that all materials that are exposed to the oral milieu are immediately covered by salivary constituents and later by oral microorganisms [16]. The surface properties of a given material such as surface roughness and hydrophobicity may influence particularly the early phases of microbial adhesion decisively, with surfaces with high surface roughness or low hydrophobicity yielding more plaque than surfaces with low surfaces roughness or high hydrophobicity in vivo [17–19]. For single strains, it has been found that bacteria with low cell surface free energy preferentially adhere to solid surfaces with low surface free energy [20–22]. The formation of complex biofilms on the surfaces of dental restorations may contribute to the occurrence of diseases such as caries, parodontopathia or denture stomatitis [23], which might coincide with restoration failure. As damage in composites may be due to the deterioration of matrix and fillers, mechanical and environmental loads, microcracking, or filler particle fracture [24], it is clear that ageing processes may influence the plaque formation on the surface of dental materials decisively.

Thus, the aim of this in vitro study was to investigate the surface properties (roughness, hydrophobicity) and adhesion of *Streptococcus mutans* to different dental composites after various artificial ageing regimes. It was hypothesized that (a) the ageing duration, (b) the ageing protocol, and (c) the tested material have a significant influence on the test variables.

2 Materials and methods

2.1 Specimen preparation

Round specimens (height 2 mm, diameter 10 mm) were prepared from five current dental composite materials (cf. Table 1) according to the guidelines provided by the manufacturers, and light cured using a conventional light polymerization device (40 s; 800 mW/cm^2; Elipar Tri-Light, 3M Espe, Seefeld, G). Prior to polymerization, specimens were covered with a transparent plastic film to prevent the formation of an oxygen inhibited layer. For each material, a total of 330 specimens was prepared.

Prior to further treatment, all specimens were smoothed using silicone carbide paper (grain 1000 and 4000, successively, Buehler GmbH, Düsseldorf, G) and a rotating grinding disc apparatus (Motopol 8, Buehler Ltd., Coventry, UK). The specimens were subsequently polished to high gloss using conventional burnishers and universal polishing paste (Ivoclar Vivadent, Schaan, FL). All specimens were stored in distilled water that was exchanged daily for 6 days prior the experiments for minimizing influences of residual monomers or toxic constituents on cell viability.

Table 1 Composite materials used in this study

Name	Class	Manufacturer	Monomer	Filler content (wt%)
Filtek Supreme XT	Composite, nano-ceramic	3M ESPE, Seefeld, G	Bis-GMA, TEGDMA, UDMA, Bis-EMA	78.5
Filtek Silorane	Composite, silorane-based	3M ESPE	Silorane (3,4-epoxycyclohexyl ethylcyclo-polymethylsiloxane, bis-3,4-epoxycyclohexyl-ethylphenylmethylsilane)	76.0
Experimental ormocer	Ormocer	Voco GmbH, Cuxhaven, G	Ormocer resin	87.0
CeramX	Composite, nano-ceramic	Dentsply DeTrey, Konstanz, G	Methacrylate modified polysiloxane, dimethacrylate resin	76.0
Quixfil	Composite	Dentsply DeTrey	UDMA, TEGDMA, di- and trimethacrylate resins, carboxylic acid modified dimethacrylate resin	85.5

2.2 Artificial ageing

The different composite specimens were randomly allotted to one of the artificial ageing protocols. Ageing simulation was carried out either by storage in ethanol (Ethanol 96%, Carl Roth GmbH + Co KG, Karlsruhe, G), artificial saliva, or distilled water for 7, 90, or 365 days (25°C, dark). Thermal cycling (6,000 cycles 5/55°C, 5 min each) was carried out in a thermal cycler (Regensburger Kausimulator, EGO, Regensburg, G), and used as reference ageing protocol.

The artificial saliva consisted of 4.1 mM KH_2PO_4, 4.0 mM Na_2HPO_4, 24.8 mM $KHCO_3$, 16.5 mM NaCl, 0.25 mM $MgCl_2$, 4.1 mM citric acid, and 2.5 mM $CaCl_2$ [25] and has been used in a previous investigation for the artificial ageing of resin specimens [26]. The pH of the artificial saliva solution was adjusted to 6.7 with 10 N HCl, and the solution was subsequently sterilized using single use filtration devices with a pore-size of 0.22 µm (Vacuflo, Schleicher & Schüll Microscience GmbH, Dassel, G) [27]. All saliva solutions were exchanged every week during the artificial ageing period.

2.3 Determination of surface roughness and hydrophobicity

Prior and after each artificial ageing treatment, surface roughness and surface hydrophobicity were measured.

Peak-to-valley surface roughness (R_a) was determined at three randomly selected spots of each specimen (two at the margins, one in central position) using a profilometric contact surface measurement device (Perthen S6P, Feinprüf-Perthen, Göttingen, G). A distance of 1.75 mm was measured in one single line scan perpendicular to the expected grinding grooves using a standard diamond tip (tip radius 2 µm, tip angle 90°) and a cut off level of 0.25.

For the evaluation of surface hydrophobicity, the surfaces of the specimens were carefully cleaned using ethanol and applicator brush tips (3M Espe, Seefeld, G), and contact angles (bidistilled water) were determined using the sessile drop method and an automated contact angle measurement device (OCA 15plus, DataPhysics Instruments GmbH, Filderstadt, G). Ten drops (0.2 µl) were analyzed on three randomly selected specimens for each material and ageing treatment; left and right contact angles were averaged.

2.4 Saliva preparation

Unstimulated whole saliva was collected by expectoration from one volunteer healthy female donor aged 25 years, who refrained from ingestion and oral hygiene for at least 2 h and gently rinsed her mouth with water prior saliva collection to minimize microbial contamination. Saliva was frozen immediately after collection, and was carefully defrosted and sterilized using single-use filtration devices (0.45 and 0.22 µm, successively) directly before the experiments.

2.5 Bacteria preparation

A frozen (−60°C) preculture of the strain *S. mutans* NCTC 10449 (DSMZ; *Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH*, Braunschweig, G) was established, and bacteria were transferred onto an agar plate and incubated at 37°C for 48 h. A single colony was incubated with sterile DSMZ-medium 92 (*Trypticase Soy Yeast Extract Medium*) at 37°C for 16 h, and subsequently kept at 4°. The day before the experiment 1 ml of *S. mutans* suspension was inoculated with 250 ml of sterile medium, and incubated for 12 h at 37°C. Cells were harvested by centrifugation (2,200 rpm, 19°C, 5 min; Hettich *Rotixa P*, Tuttlingen, Germany), washed twice with phosphate buffered saline (PBS; one tablet dissolved in 200 ml of deionized water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride (pH of 7.4 at 25°C); Sigma–Aldrich, St. Louis, MO, USA), and resuspended in the same buffer. The cell suspension was subjected to low intensity ultrasonic energy in order to disperse bacterial chains [28], and the optical density of the bacteria suspension was adjusted to 0.3 at 550 nm (*Genesys 10-S*, Thermo Spectronic, Rochester, NY, USA), which corresponds to a microbial concentration of 3.65×10^8 cells/ml [29].

2.6 Test assay

For the determination of streptococcal adherence, a modified Resazurin reduction assay was carried out as described in previous investigations [30, 31].

Fifteen specimens for each material and ageing protocol were equilibrated with ethanol, transferred to 48 well cell clusters (48 Well Cell Culture Cluster, Corning Inc., Corning, NY, USA), and the relative fluorescence intensity of each specimen prior the adhesion assay (rfi_p) was determined using an automated multidetection reader (Fluostar Optima, BMG Labtech, Offenburg, G). Subsequently, specimens were incubated either with 1 ml of PBS or 1 ml of whole saliva for the simulating an acquired salivary pellicle (ThermoForma, Marietta, OH, USA). After an incubation time of 2 h at 37°C, specimens were carefully rinsed with PBS using a standardized rinsing protocol, and incubated with 1 ml of *S. mutans* suspension and 15 µl Resazurin (Resazurin, Sigma–Aldrich, St. Louis, MO, USA) at 37°C. After an incubation time of 2.5 h, specimens were gently rinsed twice with PBS (1 ml) under standardized conditions for removing unbound bacteria, and relative fluorescence intensities after the adhesion

assay (rfi_a) were measured. Relative fluorescence intensities (rfi) were calculated according to the formula $rfi = rfi_a - rfi_p$.

2.7 Statistical analysis

All calculations and graphic display were carried out using SPSS 16.0 for Windows (SPSS Corporation, Chicago, Ill, USA). Means and standard deviations for R_a, water contact angles, and relative fluorescence intensities were calculated. Normal distribution of data was verified using the Kolmogorov–Smirnov test. Three-way analysis of variance (ANOVA) was used to analyze the influence of ageing duration (new; 7, 90, 365 days; thermal cycling), composite material and ageing medium (ethanol, distilled water, artificial saliva) on the adherence of *S. mutans* to the uncoated and saliva-coated specimens as well as on R_a and surface hydrophobicity. The Tukey–Kramer multiple comparison test was applied for post-hoc analysis. The level of significance was set to $\alpha = 0.05$.

3 Results

3.1 Surface roughness

Three-way ANOVA indicated significant influences of the composite material ($P < 0.001$), the ageing duration ($P < 0.001$) as well as the ageing medium ($P < 0.001$) on R_a; interaction effects for composite material and ageing duration as well as ageing duration and ageing medium were significant ($P < 0.001$). Exact data and results for R_a are displayed in Table 2.

At baseline, post hoc analysis showed that *Quixfil* yielded significantly higher R_a than any other material ($P < .001$). Lowest values were observed for *Filtek Silorane*, *Ceram X* and *Filtek Supreme XT*; the experimental ormocer yielded similar R_a compared to *Filtek Supreme XT* ($P = 0.849$), but significantly higher values than *Filtek Silorane* ($P = 0.009$) or *Ceram X* ($P = 0.031$). Lowest values for R_a were detected for thermally cycled specimens, which were significantly lower than at baseline ($P < 0.001$) or as all artificially aged specimens ($P < 0.001$, respectively). After artificial ageing for 7, 90, and 365 days, post hoc analysis showed a significant increase in R_a in comparison to baseline. Artificial ageing in ethanol caused significantly higher values for R_a than artificial ageing in distilled water ($P = 0.001$).

3.2 Contact angles

Three-way ANOVA showed that the composite material ($P < 0.001$) as well as the ageing duration ($P < 0.001$) had

Table 2 Surface roughness for the tested composites

Substratum	New	TC	Distilled water			Ethanol			Artificial saliva		
			7	90	7	7	7	365	7	90	365
Filtek Supreme	0.06 (0.03)	0.07 (0.02)	0.06 (0.03)	0.09 (0.05)	0.07 (0.04)	0.07 (0.04)	0.07 (0.04)	0.07 (0.04)	0.07 (0.04)	0.08 (0.02)	0.07 (0.05)
Filtek Silorane	0.04 (0.0)	0.04 (0.01)	0.06 (0.03)	0.06 (0.02)	0.07 (0.03)	0.07 (0.03)	0.07 (0.03)	0.06 (0.04)	0.07 (0.03)	0.10 (0.02)	0.09 (0.03)
Exp. ormocer	0.06 (0.02)	0.07 (0.02)	0.07 (0.04)	0.08 (0.04)	0.10 (0.02)	0.10 (0.02)	0.10 (0.02)	0.09 (0.02)	0.10 (0.02)	0.09 (0.02)	0.08 (0.02)
CeramX	0.04 (0.01)	0.06 (0.04)	0.05 (0.02)	0.06 (0.02)	0.08 (0.04)	0.08 (0.04)	0.08 (0.04)	0.07 (0.04)	0.08 (0.04)	0.07 (0.03)	0.07 (0.03)
Quixfil	0.10 (0.03)	0.09 (0.02)	0.09 (0.02)	0.12 (0.04)	0.11 (0.03)	0.11 (0.03)	0.11 (0.03)	0.11 (0.02)	0.11 (0.03)	0.13 (0.02)	0.13 (0.05)

Means (standard deviations), $n = 30$

a significant effect on water contact angles; no significant influence was found for the ageing medium ($P = 0.958$) nor the interactions. Data are displayed in Table 3.

Post hoc analysis indicated lowest water contact angles for the experimental ormocer and *Filtek Supreme XT*; contact angles for the experimental ormocer were significantly lower than values for any other material but *Filtek Supreme XT* ($P = 0.448$). Intermediate values were found for *Quixfil*, and significantly highest contact angles were found for *Ceramx X* and *Filtek Silorane*; *Filtek Silorane* yielded significantly higher contact angles than any other material ($P < 0.001$). For thermally cycled and new specimens (baseline), significantly lowest contact angles were found; significantly higher values were observed after 7, 90, and 365 days of artificial ageing.

3.3 *Streptococcus mutans* adhesion

For uncoated specimens (compare Fig. 1), three-way ANOVA indicated significant effects of the material ($P < 0.001$), the ageing duration ($P < 0.001$) and the ageing medium ($P < 0.001$) as well as their interactions on *S. mutans* adhesion. For saliva-coated specimens (compare Fig. 2), significant effects of the material ($P < 0.001$), the ageing duration ($P < 0.001$) and the ageing medium ($P < 0.001$) on *S. mutans* adhesion were observed; significant interaction effects were observed for material and ageing medium ($P < 0.001$), and ageing duration and medium ($P = 0.050$).

For uncoated specimens, lowest values for relative fluorescence intensity indicating lowest adhesion of *S. mutans* were found for *Quixfil*; similar values were detected for *Filtek Silorane* ($P = 0.530$). Intermediate values were found for *Filtek Supreme XT* and *Ceram X*, which were significantly higher than for *Quixfil* and *Filtek Silorane*. The experimental ormocer yielded significantly higher relative fluorescence intensities than any other material ($P < 0.001$, respectively), which indicates highest adhesion of streptococci. For saliva-coated specimens, post hoc analysis showed similar relative fluorescence intensities indicating similar adhesion of *S. mutans* for *Filtek Silorane* and *Quixfil* ($P = 0.931$); significantly lower fluorescence intensities indicating lower adhesion of streptococci were found for these materials than for *Filtek Supreme XT* and the experimental ormocer ($P < 0.001$, respectively). Intermediate values were found for *Ceram X*, which were significantly higher than for *Filtek Silorane* ($P < 0.023$) and significantly lower than for *Filtek Supreme XT* ($P = 0.005$) and the experimental ormocer ($P < 0.001$).

For uncoated specimens, relative fluorescence intensities increased significantly with ageing duration; lowest values were found at baseline and after 7 days, which were significantly lower than after any other ageing duration or after

Table 3 Water contact angles for the tested composite specimens

Substratum	New	TC	Distilled water			Ethanol			Artificial saliva		
			7	90	365	7	90	365	7	90	365
Filtek Supreme	75.0 (8.3)	67.7 (6.3)	79.9 (15.7)	74.1 (13.4)	85.8 (8.3)	86.1 (11.1)	79.0 (14.3)	87.3 (14.6)	86.3 (8.7)	77.9 (7.0)	78.0 (8.6)
Filtek Silorane	85.7 (5.5)	81.2 (6.8)	95.4 (7.3)	90.7 (9.9)	95.7 (10.1)	86.9 (7.6)	81.7 (18.2)	100.8 (4.7)	94.2 (4.1)	92.3 (11.9)	89.7 (8.1)
Exp. ormocer	73.6 (7.4)	62.4 (3.7)	78.2 (6.8)	76.6 (8.7)	76.7 (8.6)	76.7 (7.6)	72.0 (8.3)	84.0 (7.4)	78.2 (4.9)	72.2 (7.2)	86.0 (11.1)
CeramX	80.5 (5.9)	85.9 (3.2)	90.6 (6.7)	90.6 (11.2)	88.4 (9.9)	89.8 (10.8)	77.0 (8.9)	87.4 (5.4)	90.9 (4.7)	81.1 (9.2)	84.8 (8.8)
Quixfi	75.3 (5.0)	72.8 (9.9)	81.9 (9.3)	78.8 (8.0)	90.1 (9.4)	83.7 (10.7)	82.2 (11.1)	87.1 (11.9)	85.8 (7.4)	89.8 (11.9)	80.8 (9.1)

Means (standard deviations), $n = 9$

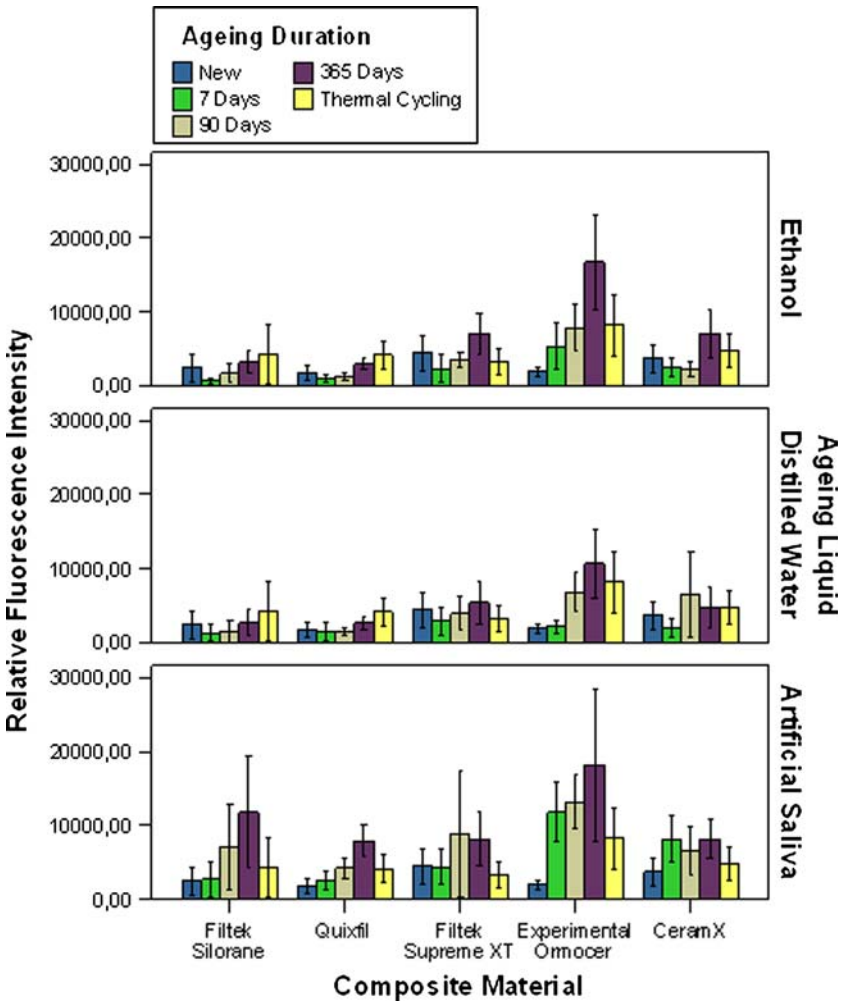

Fig. 1 Relative fluorescence intensities for uncoated composite specimens. Means and standard deviations are indicated ($n = 15$)

thermal cycling ($P < 0.001$). Similar relative fluorescence intensities were found after 90 days and after thermal cycling ($P = 0.981$); after 365 days of incubation, significantly highest relative fluorescence intensities indicating highest adhesion of *S. mutans* was found ($P < 0.001$, respectively). For saliva-coated specimens, significantly highest values for relative fluorescence intensities were found at baseline and for thermally cycled specimens; values were significantly higher than for any other material ($P < 0.001$). Significantly lower relative fluorescence intensities were found after 7 and 365 days of incubation than for any other ageing duration ($P < 0.001$); intermediate values were found after 90 days of artificial ageing.

For uncoated specimens, similar relative fluorescence intensities were found for specimens that had been stored in ethanol and distilled water ($P < 0.659$); for storage in artificial saliva, significantly higher values were observed ($P < 0.001$, respectively). Similarly for saliva-coated specimens, no significant differences in relative fluorescence intensities were found for specimens that had been stored in ethanol and distilled water ($P = 0.597$); for storage in artificial saliva, significantly higher relative fluorescence intensities were found than for storage in distilled water ($P = 0.004$) but not for storage in ethanol ($P = 0.084$).

4 Discussion

Any dental material needs to yield sufficient durability against ageing influences. However, numerous previous in vitro studies simulating clinical ageing focussed predominantly on its impact on the mechanical performance of the material [10–15]; its potential influence on microbial adhesion has largely been neglected. Thus, this in vitro study aimed to verify the influence of artificial ageing parameters on the early adhesion of *S. mutans* to dental composite materials, and to clarify the influence of ageing on the substratum surface properties. For analysis, a representative repertory of contemporary dental composite materials for the direct restoration of class II defects has been selected. At baseline, all materials were polished to high gloss using a standardized polishing regime, which

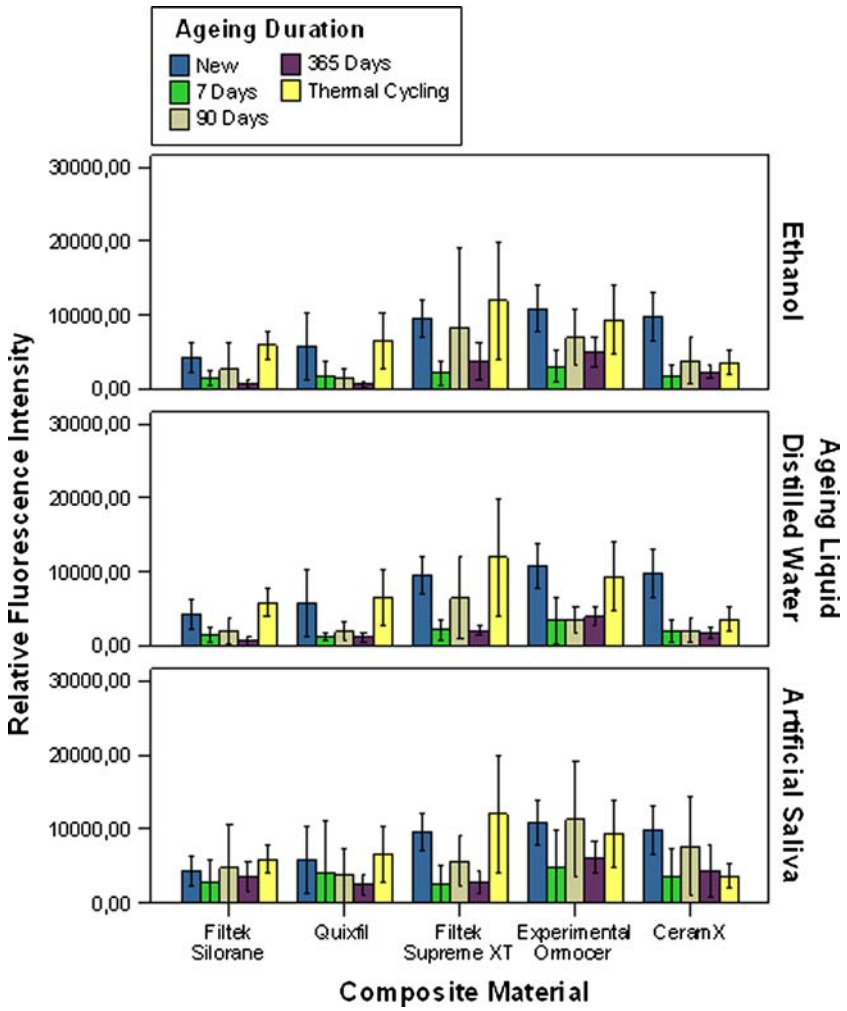

Fig. 2 Relative fluorescence intensities for saliva-coated composite specimens. Means and standard deviations are indicated ($n = 15$)

provided similar conditions for all materials prior the artificial ageing process [26, 32], and complies with the clinical procedure for finishing direct composite restorations.

In the oral cavity, dental materials are exposed to permanent humidity, and continuous exposition to saline solutions such as saliva or other aggressive liquids such as ethanol during ingestion may contribute to material degeneration. Most previous studies simulated only few aspects of the clinical ageing process over limited time spans [26], which do not address the different long-term ageing conditions in vivo satisfactorily. These considerations justify the immersion of specimens in different ageing liquids such as distilled water, artificial saliva or ethanol. However, particularly for artificial saliva it has to be borne in mind that most formulations are haphazard [33], which underlines the relevance for standardization in this matter. In addition, repeated temperature changes may take place intraorally particularly during ingestion; these circumstances have been simulated by thermal cycling. Both thermal cycling and storage in chemical liquids have widely been used in previous studies investigating the influence of artificial ageing of dental materials [14, 15, 26, 34].

The results of this study clearly support the research hypothesis, implying that artificial ageing has a decisive influence on the properties of the various materials regarding the test parameters surface roughness, surface hydrophobicity and *S. mutans* adhesion.

Surface roughness has been found to be one of the most pivotal surface properties influencing microbial adhesion [17–19], which underlines its relevance for studies on microbial adherence. In this study, storage in ethanol, artificial saliva and distilled water caused a significant increase of surface roughness, which is in well accordance with previous studies on similar topics [35–38]. This phenomenon may be due to the deterioration of the resin surface, which is caused by degradation of the filler–matrix bonds and the subsequent elution of matrix constituents. However, other studies found only marginal influences of artificial ageing on surface roughness [26], which might be attributed to different ageing behaviour of different materials chosen for analysis. Furthermore, it should be borne

in mind that not all surface defects and irregularities can be detected by profilometry as used in this study. Surprisingly, thermal cycling led to a decrease in surface roughness; further analysis is necessary to clarify this aspect. Evaluating the influence of surface roughness on dental plaque formation, Bollen and co-workers detected a threshold value at 0.2 μm, implying that higher values for surface roughness cause a significant increase in plaque formation [18]. For any tested material, values for surface roughness were far below this threshold value both prior as well as after artificial ageing. Thus, it is unlikely that the differences in microbial adhesion can be attributed to variations in surface roughness; in addition, the findings indicate that the surface roughness of modern dental composite materials increases only slightly with prolonged clinical service. Generally, the conventional composite material (*Quixfil*) yielded highest surface roughness, which is probably due to its higher content of large filler particles. In contrast, similar surface roughness was detected for the silorane-based composite (*Filtek Silorane*), *CeramX* and the nano-filled composite (*Filtek Supreme XT*), which indicates that these materials yield similar ageing behaviour with regard to this aspect.

Apart from surface roughness, surface hydrophobicity has been found to be another pivotal factor influencing microbial adhesion to oral interfaces [17–19]. It is generally accepted that surfaces with water contact angles higher than 90° are referred to as hydrophobic, whereas surfaces with water contact angles lower than 90° are described as hydrophilic. Data gathered from this investigation showed that the duration of ageing had a significantly more pronounced effect on water contact angles than the ageing medium, which was negligible. For new and thermally cycled specimens, similar hydrophobicity was found, which suggests that there is no increased water absorption of the various composite materials during thermal cycling, which would have been indicated by a significant hydrophilization of the test surfaces. For any ageing medium, water contact angles increased with ageing duration, showing lowest values at baseline, and highest values after storage for 1 year, which indicates a decisive hydrophobization of the resin surface. These findings comply with a previous investigation from our department, observing significant decreases in surface free energy of composite materials after artificial ageing for 6 months which coincides with more hydrophobic surface properties [38]. However, in another study investigating the performance of prosthetic resins only few significant alterations in surface free energy after artificial ageing were found [26], which underlines the relevance of substratum properties and its durability against ageing influences. In addition, it has to be borne in mind that due to the inhomogeneous composition of the surface of dental composites it is difficult to render reproducible contact angle measurements. It has been reported that ethanol has a similar solubility coefficient as Bis-GMA [39], which implies that immersion in ethanol fosters the elution of unpolymerized monomers; however, in this study no significant differences of the various ageing media on the surface properties could be detected. Generally, the silorane-based composite (*Filtek Silorane*) showed significantly highest values for surface hydrophobicity. This phenomenon is most likely due to its hydrophobic siloxane backbone [6, 7], which causes more hydrophobic surface properties than conventional methacrylate-based matrix components; similar results have been reported in previous investigations [38]. The generally lower surface hydrophobicity that has been detected for the experimental ormocer or the nano-filled composite (*Filtek Supreme XT*) indicates a more hydrophilic matrix–filler system than for the other composite materials.

In vivo, dental restorations are immediately covered by a thin film of salivary constituents, which is commonly referred to as the acquired pellicle [40]. This layer may have a significant influence on microbial adhesion, as it causes a levelling of originally distinct substratum surface hydrophobicities [41, 42], and may furthermore influence microbial adhesion by direct interaction of pellicle constituents with oral microorganisms. With regard to these considerations, the authors refrained from the investigation of contact angles for saliva coated specimens; future studies might, however, analyze the protein composition of the pellicle layer on the surface of dental composite materials after different artificial ageing protocols, and relate their findings directly to the adhesion of bacteria. For simulating the influence of the acquired pellicle on microbial adhesion, artificially aged specimens have been exposed to human saliva for 2 h prior the *S. mutans* adhesion assay. It has been reported that the salivary pellicle reaches its maximum thickness after 2 h [43, 44], which justifies the approach used in this study. Additionally, for evaluating the influence of the bare substratum surface after artificial ageing, specimens have been exposed to *S. mutans* suspension directly without prior exposition to saliva. *S. mutans* has been found to be one of the major causative agents for dental caries [45], and though it is no typical early-colonizing bacterium it has been detected in initial dental plaque [46]; these considerations justify its selection as test strain. In addition to the surface properties of the substratum surface it has been reported that the surface properties of the bacteria play a decisive role in the adhesion to solid surfaces, too: with regard to this aspect, it has been found that bacteria with low surface free energy preferentially adhere to solid surfaces featuring low surface free energy, which corresponds to hydrophobic surface properties [20–22]. For the *S. mutans* strain that has been used in this study, hydrophobic surface properties have been reported [47], which

corresponds to low cell surface free energy; thus, increased adhesion to hydrophobic surfaces had to be expected. However, with regard to this aspect it should be borne in mind that ionic strength may influence the adhesion of bacteria decisively. For PBS, it has been reported that the cell surface properties (surface hydrophobicity, zeta potential) of streptococci may be different for high and low ionic strength PBS [48]; these aspects might be investigated more thoroughly in future investigations. For both uncoated and saliva-coated specimens, the silorane-based composite (*Filtek Silorane*) and the conventional composite (*Quixfil*) showed lowest values for *S. mutans* adherence. These findings comply with a previous study on microbial adhesion to several composite materials, reporting lower adhesion of streptococci to a silorane-based composite than to several conventional methacrylate-based resin composites [38], but complies only in parts with the thermodynamic approach for the explanation of microbial adhesion to oral interfaces as the silorane-based composite yielded significantly higher surface hydrophobicity than the conventional composite. However, previous studies from our department have demonstrated that the thermodynamic model does only to some extent coincide with early microbial adhesion to dental materials surfaces [30], and other researchers agree that physicochemical approaches based on overall surface properties for the explanation of bacterial adhesion to solid surfaces frequently fail [48]. With regard to this aspect, it should be borne in mind that this approach has been developed for model surfaces; the surface of dental composite materials is usually more complex [49], and may be very inhomogeneous due to fractions of filler and different matrix constituents that are present of the surface of a composite material. In addition, these findings support the theory that the original surface properties are transferred even through a surface protein layer [50], and still influence microbial adhesion.

Surprisingly, a different influence of artificial ageing duration was observed for uncoated and saliva-coated specimens. As expected from previous studies with *Candida albicans* [26], prolonged artificial ageing led to an increase in *S. mutans* adhesion for uncoated specimens, whereas, for saliva-coated specimens the adhesion of streptococci decreased after artificial ageing. With regard to this aspect, it might be possible that the experimental pellicle led to a substantial modification of surface hydrophobicity, which might have caused unfavourable conditions for *S. mutans* adhesion. It has been reported that the morphology of an intraorally formed pellicle layer is influenced significantly by the type of filler [51], which might have a significant influence on the subsequent adhesion of microorganisms. With regard to this aspect, it is clear that further studies are necessary to clarify these aspects, for instance by clarifying potential differences in the pellicle composition of artificially aged materials. For both uncoated and saliva-coated specimens, significantly lowest values for *S. mutans* adherence were observed after 7 days of artificial ageing; this phenomenon might be due to balance between the progressive elution of residual monomers [52, 53], which might have an inhibitory effect on the viability of microorganisms, and the influence of the ageing process. Surprisingly, intermediate values for relative fluorescence intensities were found for saliva-coated specimens after 90 days, and significantly lower values were detected after 365 days, which indicates lower adhesion of *S. mutans*. These findings require further discussion, and underline the need for thorough investigation of pellicle composition on artificially aged composite specimens.

Concerning the influence of the ageing medium, the findings of this study indicated higher adhesion of streptococci to specimens that had been immersed in artificial saliva compared to those that had been immersed in ethanol or distilled water. Surface roughness and hydrophobicity could not explain this phenomenon sufficiently. However, the artificial saliva that has been used in this study was supplemented with various, highly concentrated salts; most likely, these led to substantial changes in the surface chemistry of the resin materials, causing a significant increase in *S. mutans* adhesion. The artificial saliva formulation that has been used in this study [25] has been applied by Söderholm and co-workers for investigating the filler leachability of dental composite materials, finding significantly higher filler leachability for specimens that had been stored in artificial saliva compared to those that had been stored in ethanol [54]; these findings underline the aggressive character of artificial saliva. Overall, these findings highlight the relevance of the ageing medium for the outcome of studies dealing with artificial ageing of dental composite materials, and require further analysis in future studies, for instance by means of energy-dispersive X-ray spectroscopy for determining the composition of surface constituents.

5 Conclusion

Within the limitations of an in vitro study, it can be concluded that modern dental composite materials based on silorane or ormocer technology may yield similar ageing behaviour than conventional or nanofilled composite materials with regard to the adhesion of *S. mutans*. However, the findings indicated significant influences of the artificial ageing duration and the ageing medium, which underlines the need for sufficient standardization of artificial ageing protocols for the analysis of dental materials.

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