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Effect of water storage of E-glass fiber-reinforced composite on adhesion of *Streptococcus mutans*

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Abstract

This study investigated the effect of water storage of fiber-reinforced composite on the adhesion of *Streptococcus mutans* (*S. mutans*) and its ability to stay adhered and multiply on the FRC. The materials (E-glass fibers and denture base polymer) were stored in water for 14 or 30 days or left dry. Water contact angles of the materials before and after water storage were determined. Test specimens, with or without parotid saliva or serum pellicle, were incubated in a suspension of *S. mutans* allowing initial adhesion to occur. Bacterial adhesion and multiplication was studied using scanning electron microscopy. Contact angles of both materials were significantly reduced after water storage indicating an increase in surface free energy. When studied without a surface pellicle, water storage significantly increased adhesion of *S. mutans* to both glass and polymer. Saliva coating of the materials resulted in higher degree of adhesion to glass fibers in comparison with polymer and after 14 days water storage glass bound over twice as much *S. mutans* cells than the polymer matrix. Bacterial growth and biofilm formation occurred equally on both materials. The results of this in vitro study suggest that in order to avoid the possible increase in *S. mutans* adhesion, the reinforcing glass fibers should be covered with the matrix polymer of the composite.

Keywords: Adherence; *Streptococcus mutans*; Fibers; Reinforcements; Dental materials; Fiber-reinforced composite

1. Introduction

The use of fiber-reinforced composites (FRC) in dentistry has increased during recent years. Their mechanical properties, especially fatigue resistance, have been shown to be superior in applications like removable dentures [1]. The possibility to save tooth tissue and achieve metal free restorations has made them also an alternative in the choice of material in fixed prosthodontics [2]. Glass fibers, including electrical glass (E-glass) fibers are preferred in dental applications due to their aesthetic appearance and good adhesion to matrix polymers via silane coupling agents. Adhesion and colonization of certain oral pathogens has been shown to be harmful to the teeth and periodontal tissues [3]. All biological and non-biological solid surfaces in the oral cavity provide the caries-associated *Streptococcus mutans* (*S. mutans*) a surface to adhere to and multiply on. The reinforcing fibers used in FRCs are usually covered with the matrix polymer or particulate filler composite. However, when the FRC appliance is adjusted into occlusion or polished, or is designed to be very thin, the fibers are exposed and come in contact with oral microbes. In our previous studies conducted with E-glass — polymethylmethacrylate composite we found that the salivary pellicle formed on FRC promoted adhesion of *S.mutans* on glass fibers in comparison with the polymer matrix [4]. These studies were conducted using only newly polymerized FRCs as test materials.

In the oral cavity FRCs are exposed to an aqueous environment. Water is known to diffuse into the matrix polymer causing plasticizing of the polymer and weakening the mechanical properties of FRCs [5]. On the other hand, a newly cured polymer contains residual monomers, which leach out during storage in water [6]. Elements such as alkali metals are known to leach out of the E-glass surface when it is exposed to aqueous conditions [7]. These phenomena may change the surface properties of FRCs and affect interaction of microorganisms with the surface.

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Initial bacterial adhesion is controlled by several physico-chemical factors, like van der Waals and electrostatic forces. Surface-free energy and hydrophobicity of the adherent surface also play a role in the process. The initial adhesion of the caries-associated *S. mutans* is predominantly defined by electrostatic forces [8]. Salivary proteins mediate adhesion in the presence of an acquired pellicle [9]. The initial adherence of bacteria is followed by colonization and formation of a biofilm. In the presence of sucrose *S. mutans* cells use glucosyltransferases and extracellular polysaccharides as mediators in the biofilm formation [10]. Biofilms have been suggested to be the preferred method of survival in nature [11]. Bacteria coadhering provide each other more adhesion sites thus strengthening the biofilm that in part protects the bacteria from detaching.

The aim of this in vitro study was to determine the effect of water storage of FRC on the adhesion of *S. mutans* and its ability to stay adhered and multiply on the FRC.

2. Materials and methods

2.1. Materials

The materials used in this study are listed in Table 1. For the bacterial adhesion tests, nine test specimens were prepared from polymer preimpregnated unidirectional E-glass fiber reinforcement embedded in auto polymerized denture base resin. The preparation of test specimens has been previously described [4]. Wet ground and polished test specimens (grit no. 800 and 2400; Struers A/S, Copenhagen, Denmark) were sterilized in ethylene oxide. The control specimens were left dry and stored in a desiccator for 30 days. The water storage of the specimens was carried out in distilled water at 37°C for either 14 or 30 days. The water stored specimens were transferred into the precoating solutions immediately after removal from water storage.

2.2. Initial adhesion experiment

The method for precoating of the test specimens with protein solutions and the method used to study bacterial adherence have been previously described in detail [4]. After the storage one test specimen of each water-storage group was coated with saliva, one with serum and one was left uncoated. Stimulated human fresh parotid saliva (1:1 in phosphate-buffered saline (PBS; Orion Diagnostica, Espoo, Finland) and human serum (1:5 in PBS) were used as the protein solutions for precoating. Immediately after precoating, the test specimens were subjected to adherence tests. The test organism used in this study was *S. mutans* (NCTC 10449). Log phase cells were used to prepare a suspension in PBS at a concentration of $A_{660} \sim 0.5$, corresponding approximately to 7×10^8 colony-forming units. The suspension was subjected to ultrasonic treatment to disrupt long streptococcal chains. The test specimens were incubated in the bacterial suspension using stirring at room temperature for 30 min. The specimens were thereafter gently rinsed in PBS and prepared for scanning electron microscopy (SEM). The results were verified by repeating each experiment three times.

2.3. Growth experiment

In these experiments control specimens and specimens water-stored for 14 days were used both with and without precoating with parotid saliva. After the initial adhesion described above the specimens with the attached *S. mutans* cells were gently rinsed in PBS and subsequently transferred into brain heart infusion (BHI; Unipath Ltd, England) medium with 5% added sucrose (w/v). The specimens were incubated in the medium at 37°C temperature for up to 7 h.

2.4. Scanning electron microscopy

All specimens were fixed with 0.25% glutaraldehyde and dried with an ascending series of ethanol. The

Table 1
Materials, their compositions and manufacturers, used in this study

Code	Brand name	Manufacturer	Composition (%)	Batch
GF	Glass fiber-prepreg	StickTech Ltd, Turku, Finland	Silanized electrical glass SiO_2 55; CaO 22; Al_2O_3 15; B_2O_3 6	1970129-R-017
PP	Palapress	Hareus Kulzer GmbH, Wehrheim, Germany	PMMA[a]/BDMA[b]	1967/949
E-glass	Bulk E-glass	Ahlström, Karhula, Finland	Electrical glass SiO_2 55; CaO 22; Al_2O_3 15; B_2O_3 6	7149-1

[a] Polymethylmethacrylate.
[b] Butanedioldimethacrylate.

specimens were then covered with a layer of carbon. Five SEM micrographs (×2000) from the fiber-rich area and five from the polymer matrix area were taken of each specimen. For the initial adhesion experiment the adhered bacteria were counted per mm^2.

2.5. Contact angle measurements

Six test specimens ($10 \times 8 \times 2$ mm) were made from bulk E-glass and six from auto polymerized denture base resin (Palapress). The specimens were wet ground and polished (grit no. 800 and 2400; Struers A/S, Copenhagen, Denmark). Half of the glass and polymer specimens were stored in water for 14 days and half in a desiccator as described above. The contact angle measurements were performed with an optical contact angle meter (CAM200, KSV Instruments Ltd, Helsinki, Finland) using water as the wetting agent. Three drops were measured from each polymer specimen and two from each glass specimen. On the glass specimens, due to the hydrophilicity of glass, the drops cover a larger area and only two drops could be fitted.

2.6. Statistical analyses

Statistical analyses were performed with SPSS for Windows (Rel. 8.0.1, 1998, Chicago; SPSS Inc.). The data was first subjected to one-way ANOVA. Subsequent multiple comparisons were conducted using Tukey's post hoc analysis. The level of statistical significance was considered to be 0.05.

3. Results

The highest number of adhered *S. mutans* cells was observed on saliva-coated specimens, particularly on the glass fibers (GF). Intermediate number of adhered bacteria was seen on uncoated materials and the lowest number on the serum-coated specimens. (Fig. 1).

Among the uncoated materials lowest number of adhered bacteria was seen on control specimens that were kept dry. Water storage of the test specimens resulted in an increase in bacterial adhesion to both GF ($p = 0.002$) and polymer matrix (PP) ($p < 0.001$). On the dry specimens adhesion of *S. mutans* was similar to GF in comparison with PP. However, more adhered bacteria was seen on PP compared to GF, when the specimens were stored in water for 14 ($p = 0.028$) or 30 days ($p = 0.022$). (Fig. 1).

For all saliva-coated specimens a statistically significant difference with higher numbers of adhered *S. mutans* cells to GF in comparison with PP was observed. The difference was most distinctive on the 14-day water-stored specimens ($p < 0.001$) (Fig. 2A). For the saliva-coated materials water-storage significantly increased adhesion on *S. mutans* to GF in comparison with PP. The strongest increase was seen at the 14-day time point ($p = 0.002$) and a slightly smaller increase at 30-day time point ($p = 0.017$) (Fig. 1).

Serum coating resulted in very low numbers of adhered cells to all materials and no statistically significant differences could be seen between materials or storage methods. (Fig. 1).

In the growth experiment the number of adhered cells stayed the same on both materials. At two hours time

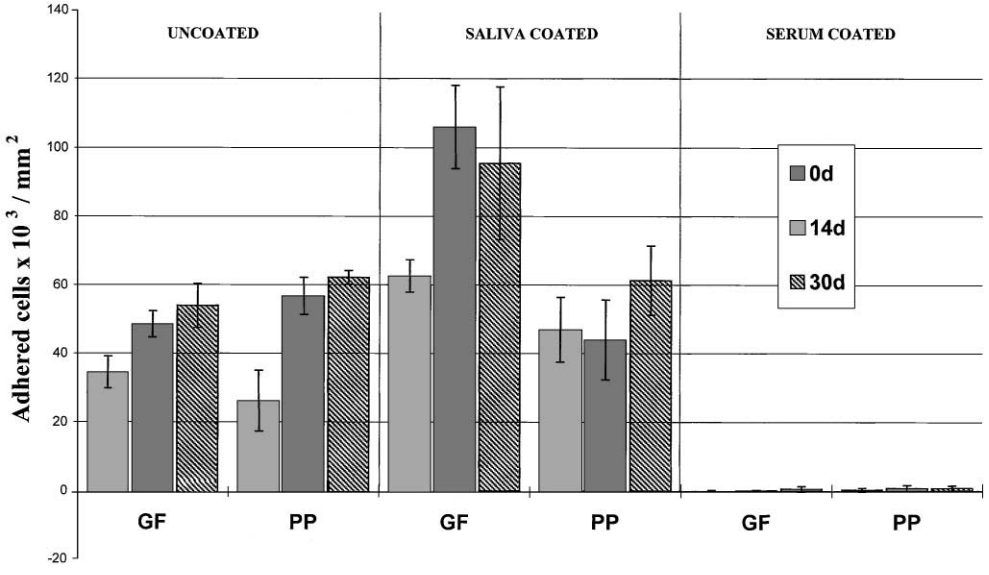

Fig. 1. Number of adhered *S. mutans* cells on glass fibers (GF) and denture base polymer (PP) with and without surface precoating with saliva or serum before (0 d) and after water storage of 14 (14 d) and 30 days (30 d). The figure shows the results of a single representative experiment of a series of three repeated experiments. The bars indicate the mean numbers ± standard deviation of five SEM fields.

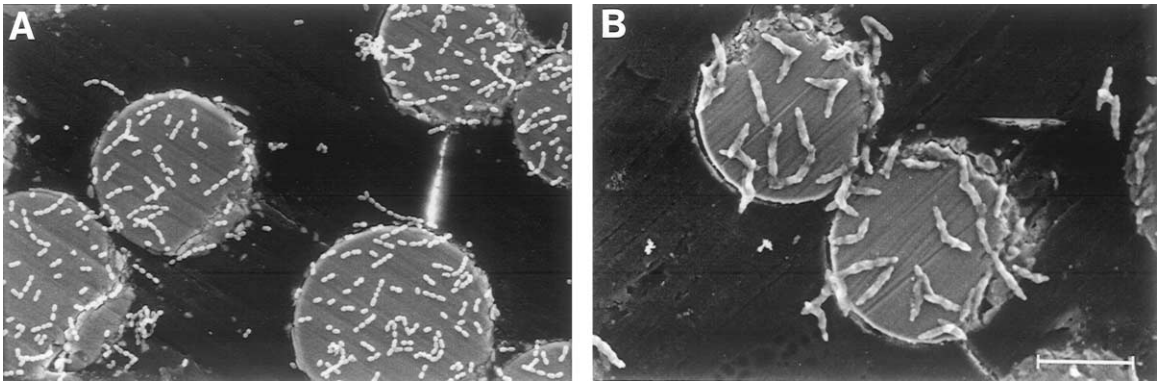

Fig. 2. Scanning electron micrographs of fiber-reinforced composite (FRC) surface with adhered *S. mutans* cells. (A) Initially adhered *S. mutans* cells on saliva-coated FRC stored in water for 14 days. (B) The initially adhered *S. mutans* cells are allowed to grow in a BHI medium with added sucrose for 2 h. Original magnification: ×2000. The bar corresponds to 10 μm.

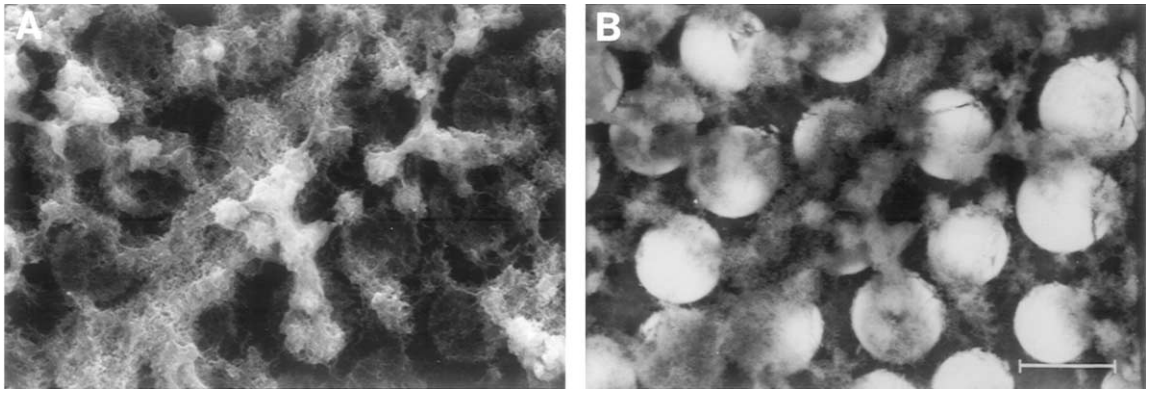

Fig. 3. Scanning electron micrographs of water stored (14 d) and saliva-coated FRC with a *S. mutans* biofilm. After initial adhesion the specimens were incubated in BHI medium with added sucrose for 7 h. (A) Picture taken using secondary electron detector. (B) Picture taken using back-scattered electron detector to visualize the fibers underneath the bacterial layer. Original magnification: ×1000. The bar corresponds to 20 μm.

Table 2
Water contact angles of denture base polymer (PP) and E-glass before and after water storage. Mean values (±standard deviation). Effect of water storage was tested using one-way ANOVA

	N	Dry stored	Water stored	*p*-value
PP	9	73.2 (±2.1)	68.8 (±4.7)	0.026
E-glass	6	33.2 (±2.3)	26.2 (±4.2)	0.005

point, cells attached to both GF and PP were dividing (Fig. 2B) and at 7 h time point the bacterial biofilm equally covered both materials of the composite specimens (Fig. 3).

The water contact angles obtained from dry and water stored specimens are shown in Table 2. The contact angles for polymer specimens were more than twice as big than the contact angles for glass specimens. Contact angles of both materials were significantly reduced as a result of water storage.

4. Discussion

The present study investigated *S. mutans* binding ability of the materials used in dental fiber-reinforced composites before and after water storage. Streptococci can adhere to all solid surfaces in the oral cavity. The adhesion mechanisms of streptococci to clean solid substrata have been extensively studied and a variety of adhesion mechanisms have been reported, including physico-chemical surface characteristics such as surface charge and hydrophobicity. In the case of the caries-associated *S. mutans* the initial adhesion seems to be based mainly on electrostatic forces [8]. Interaction with the salivary proteins and glycoproteins via surface adhesins is the primary binding mechanism to a salivary pellicle, present on all oral surfaces [9]. We observed highest numbers of adherent bacteria on saliva-coated materials. Parotid saliva contains *S. mutans* adhesion-associated agglutinins and promotes adhesion of *S. mutans* more in comparison with whole saliva or submandibular–sublingual saliva [12,13]. All serum-coated

specimens showed very little binding of *S. mutans*. Other authors have also reported this observation of a strong decrease in bacterial adhesion with a serum pellicle [14–16]. Serum albumin has been reported to act as a blocking agent for bacterial adhesion [17].

When the materials were studied without surface coating, it was observed that on the water-stored specimens there were significantly more adhered *S. mutans* cells than on the dry specimens. The increase in bacterial adhesion as a result of water storage was seen on both materials and there were no differences in bacterial adhesion between GF and PP. This observation is in accordance with our previous results [4]. The increase in adhesion on polymer surfaces may be the result of decrease in residual monomer release from the polymer during time. An autopolymerized denture base acrylic contains residual monomers, mainly methylmethacrylate, part of which is leached out of the material to an aqueous medium [18,19]. Methylmethacrylate and its oxidation product formaldehyde are the main substances released from these polymers [20]. The formaldehyde concentrations found from denture base polymers have been reported to be cytotoxic [6]. Most of the release of residual monomers occurs within 24 h after immersion of the material [6,19,21]. Small amounts of monomers continue to be released for a longer period of time. Simultaneously with the releasing of residual monomers, water molecules are absorbed in the spaces between the polymer chains. Leaching of residual monomers and low water saturation of polymer matrix may interfere with bacterial adhesion on dry-stored polymer surfaces. After 14-day water storage most of the residual monomer has been released to the storage medium and bacterial adhesion may take place in greater number. Increase in bacterial adhesion was seen on glass surface without pellicle as well. An aqueous environment can cause corrosion effect in the surface of glass fibers [7]. According to our unpublished results, some alkali metals leach from E-glass surface during water storage. This may change the surface more favorable to adhesion in terms of surface area and chemical reactivity.

GF seemed to bind more *S. mutans* than PP in all saliva-coated specimens (Fig. 1). The difference in adhesion to saliva-coated GF and PP in dry stored specimens is in accordance with our previous findings [4]. The greatest difference was however observed on the 14-day water stored specimens when adhesion to GF increased clearly while adhesion to PP remained on the previous level (Fig. 2A). On pellicle-coated surfaces adhesion of *S. mutans* is mediated by salivary components adsorbed to the surface. As we have previously concluded, saliva pellicle formed on glass fibers seems to promote adhesion of *S. mutans* in comparison with the polymer matrix. This promoting effect seems to be even stronger on water stored GF. It is possible that the changes in the surface of E-glass that occur during water storage account for this difference.

The contact angle measurements conducted with E-glass and denture base polymer showed a decrease in water contact angles i.e. an increase in surface wettability after water storage (Table 2). The change was seen on both materials. Low contact angles are an indication of high surface-free energy whereas, high contact angles indicate low surface-free energy [22]. Substrates with high surface-free energy have been reported to bind more bacteria in the presence of an acquired pellicle in comparison with surfaces of low energy [23,24]. On an originally high surface-free energy substrate, like glass, this increase in surface wettability and surface-free energy caused by storage in water may account for the increase in pellicle mediated bacterial adhesion. This phenomenon, however, needs to be further investigated before conclusions can be made. Surprisingly, only a slightly increasing trend but no significant changes in bacterial adhesion to saliva-coated PP were seen as a result of water storage. The masking effect of a saliva pellicle [25] might account for this. On the other hand, there is evidence that a given microorganism adheres differently to a hydrophilic substrate than to a hydrophobic substrate [26].

Since the initial adhesion of *S. mutans* on the 14-day water-stored specimens seemed to clearly favor the glass surface, we also studied growth of *S. mutans* on these specimens. Some substrates, for example, bioactive glass, may provide bacteria a surface for colonization but not permit biofilm formation. Stoor et al. in their study on *Haemophilus influenzae* adhesion to the bioactive glass S53P4 reported that, although initial adhesion took place, after 8 h incubation bacteria had detached the surface and no biofilm formation was observed [27]. Our results of the growth experiments however showed that the studied materials seem to neither inhibit nor promote bacterial adhesion. The initially adhered *S. mutans* cells stayed adhered and were able to multiply and grow on both PP and GF surfaces.

As a conclusion, water storage of E-glass fiber — denture base polymer composite increased the initial adhesion of *S. mutans* to both materials of the composite when studied without a surface pellicle. A saliva pellicle formed on glass fibers favored the adhesion of *S. mutans* in comparison with the polymer matrix. Water storage of the composite increased the *S. mutans* adhesion promoting effect of parotid saliva pellicle formed on glass fibers. The possible increase in *S. mutans* adhesion caused by the glass fibers can be avoided by modifying the surface properties of glass fibers or by covering the fibers with the matrix polymer of the composite.

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