Adherence of *Streptococcus sanguinis* and *Streptococcus mutans* to saliva-coated S-PRG resin blocks

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This in vitro study performed elemental analysis of the ions absorbed into the salivary coat covering the surfaces of S-PRG resin blocks and assessed the adherence of *Streptococcus sanguinis* and *Streptococcus mutans* to these saliva-coated S-PRG resin blocks. Elemental analysis of ions absorbed into the salivary coat of resin blocks exposed to the saliva was performed using an inductive coupled plasma atomic emission spectrometer and the fluoride electrode method. Quantitative adherence of radio-labeled test bacteria to the resin blocks was determined. As the results, the saliva-coated S-PRG resin showed significantly greater amounts of absorbed B, Al, Si, Sr, and F than the saliva-coated unfilled resin. It was of particular significance that the salivary coating of the S-PRG resin reduced the adherence of *S. mutans* to this resin. However, in the case of *S. sanguinis*, no significant difference in adherence could be recognized between saliva-coated S-PRG resin and saliva-coated unfilled resin.

**Keywords**: Oral streptococci, Inhibition of adherence, S-PRG filler, Release of inorganic elements, Resin composite

**INTRODUCTION**

Dental plaque, commonly referred to as an acquired pellicle, does not form directly on the surface of teeth and restorative materials. The composition of dental pellicles is mainly derived from the selective adsorption of salivary proteins. Most of the interfacial films are dominated by glycoproteins as the first spontaneously acquired conditioning layer known as the pellicle. Subsequently, bacteria and bacterial products are adsorbed onto this organic layer, which is then transformed into the dental plaque. The presence of salivary components can influence the adhesion process, by forming a conditioning film on the biomaterial surface and by interacting with the bacteria. In the process of dental plaque formation, early colonizers, including *Streptococcus sanguinis*, *Streptococcus oralis*, and *Streptococcus mitis*, adhere to the salivary coat covering the surfaces of teeth and restorative materials. This initial adhesion is an important step in dental plaque formation, as it may influence the composition of the mature dental plaque.

The surface properties of resin composite materials related to bacterial adhesion and dental plaque formation are affected by the components of the material itself. Coating by saliva may reduce the adsorption of oral bacteria onto restorative resin composites as compared with that onto uncoated resins. S-PRG resin, namely, a resin composite containing Surface Pre-Reacted Glass-ionomer (S-PRG) filler, has unique surface properties compared with other resin composites used in the oral cavity. The manufacturer has classified it as a GIOMER, as it has the characteristics of both a resin composite and a glass ionomer cement. The S-PRG filler particles are produced by using S-PRG technology. With this technology, a glass-ionomer phase is formed on glass particles through the reaction of fluoro-boro-alumino-silicate glass with a polycarboxylic acid in the presence of water. S-PRG filler has a fluoride release and recharge ability equivalent to those of glass ionomer cement. Moreover, the S-PRG filler also releases inorganic elements such as Al, Sr, Na, F, SiO32-, and BO33-. Although the mechanism of ion release from S-PRG filler was not completely understood, it was believed to have occurred because of the presence of a glass ionomer phase (gel phase of the glass core that based on the reaction between polyacid and glass powder) around the glass core filler. A highlight of our previous study was that S-PRG resin exhibited an anti-plaque quality as well as characteristics appropriate for caries treatment. We proposed that the pellicle associated with S-PRG resin is composed of several salivary constituents and some S-PRG inorganic filler components. Therefore, the release of inorganic elements uniquely contained in S-PRG resin might be related to the inhibitory action of this resin toward the dental plaque. The aim of this in vitro study was to conduct elemental analysis of the ions absorbed into the salivary coat covering the surfaces of S-PRG resin and to assess the adherence of *S. sanguinis* (one of the initial adhered bacteria in the oral cavity) and *S. mutans* (cariogenic bacteria) to saliva-coated S-PRG resin.

**MATERIALS AND METHODS**

**Preparation of specimens**

Two experimental resin composites were used in this study, S-PRG resin composite (containing 24 wt% Bis-GMA, 16 wt% TEGDMA, 40 wt% S-PRG filler, and 19.2 wt% glass ultra filler) and an unfilled resin as a control (60 wt% Bis-GMA and 40 wt% TEGDMA). Each specimen was individually introduced into a Teflon
mold (4×4×1 mm). These molds were then pressed from both sides with plastic strips and a slide glass, and light-cured for 40 s with a visible light curing unit (Coltolux 4, Colten/Whaledent, Mahwah, NJ, USA). The light-cured specimens were then removed from their mold and used as test specimens. Each specimen was stored in distilled water at 37°C for 24 h. Polishing of the surfaces was performed in distilled water with a 1,000-grid silicon-carbide paper for the removal of the non-polymerization layer and the surface appearance of S-PRG filler. After having been polished, the test specimens were washed in an ultrasonic water bath for 2 min and rinsed twice with distilled water. All specimens were wiped off the surface with ethanol raw cotton.

Preparation of saliva for coating of samples
Before the adherence experiments, some specimen blocks were exposed to sterile human saliva. Saliva was collected from 5 healthy subjects (male, 25–28 years old) who had given informed consent to participate in this study, which was approved by the Asahi University Review Board (No. 23114). The whole saliva was collected by expectoration after the subject had chewed a piece of paraffin. The whole saliva samples were sonicated (1 min, 30 W), filtered through a 70-µm filter, and clarified by centrifugation (12,000 g, 20 min, 4°C). The supernatant was filtered through a low-protein-binding filter (pore size of 0.45 µm; Millipore, USA). The sterilized saliva was stored at 4°C. Before use, the pH was adjusted to 7.1–7.3 with phosphate buffer (0.067 mol/L Na2HPO4, 0.067 mol/L KH2PO4)12).

Measurement of B, Al, Si, Sr, and F absorbed into the salivary coat covering the surfaces of S-PRG resin blocks
Experimental resin blocks (S-PRG resin or unfilled resin, n=5 per group) were immersed in a 10-mL volume of the above-mentioned whole saliva at 37°C (shaking at 120 times/min) for 24 h, after which 7 mL of 2% sodium dodecyl sulfate (SDS) was added to remove the salivary coat from the resin blocks. Then, the resin blocks were removed from the solution. Elemental analysis of ions (BO3−, Al3+, SiO32−, Sr2+, F−) present in the whole saliva, SDS solution only, and of those in the salivary coat, was performed by using an inductive coupled plasma (ICP) atomic emission spectrometer (ICP-ICPS-8000, Shimadzu, Kyoto, Japan) and the fluoride electrode method (fluoride electron: Model 9609BN, pH/ion meter: Model 720A, Orion Research). The analysis by use of an ICP atomic emission spectrometer (ICP-AES) was conducted after preparing the standard calibration curves corresponding to each element. The ICP-AES operating conditions are summarize in Table 1. Fluoride was also analyzed by using a fluoride ion electrode method after preparing calibration curves (standard solution concentration: 0.1, 1, 5, 10 ppm). An ionic-strength adjuster (TISAB III, Orion Research, Boston, MA, USA) was added in the proportion of 0.1 mL of the adjuster to 1 mL of test liquid. Mean and SD were calculated. All numerical data were statistically analyzed by using Student’s t-test (p<0.05).

Table 1 Operating conditions for ICP-AES

<table>
<thead>
<tr>
<th>Plasma condition</th>
<th>Power</th>
<th>1.2 kW (27.12 MHz)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma gas flow rate</td>
<td>Ar 1.2 L/min</td>
</tr>
<tr>
<td></td>
<td>Carrier gas flow rate</td>
<td>Ar 0.7 L/min</td>
</tr>
<tr>
<td></td>
<td>Coolant gas flow rate</td>
<td>Ar 14.0 L/min</td>
</tr>
<tr>
<td>Observation height</td>
<td>11 mm above work coil</td>
<td></td>
</tr>
<tr>
<td>Nebulizer</td>
<td>Coaxial type</td>
<td></td>
</tr>
<tr>
<td>Spray chamber</td>
<td>Cyclone type</td>
<td></td>
</tr>
<tr>
<td>Spectrometer</td>
<td>Czerny-turner mounting (f=1 m)</td>
<td></td>
</tr>
<tr>
<td>Integration time</td>
<td>5 s</td>
<td></td>
</tr>
<tr>
<td>Repetition</td>
<td>3 times</td>
<td></td>
</tr>
<tr>
<td>Wavelength</td>
<td>Al: 396.153 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B: 249.773 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Si: 251.612 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sr: 407.771 nm</td>
<td></td>
</tr>
</tbody>
</table>

Quantitative adherence of radio-labeled bacteria
The test bacteria, *Streptococcus sanguinis* (ATCC 10556) or *Streptococcus mutans* (ATCC 25175), were inoculated into 150 mL of trypticase soy broth (BBL Microbiology System, Cockeysville, MD, USA) containing 0.5% yeast extract (Difco Laboratories, Detroit, MI, USA). Then, 20 mL of 74 kBq/mL of [methyl-3H]-thymidine (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for radio-labeled *S. sanguinis* or 20 mL of 7.4 kBq/mL of [methyl-14C]-thymidine (American Radiolabeled Chemicals, St. Louis, MO, USA) for radio-labeled *S. mutans* was added, and the cells were thereafter incubated anaerobically at 37°C for 18 h. The suspensions were centrifuged at 8,000 g at 4°C for 15 min with 0.05 M phosphate buffer saline (PBS; pH7.0), and the radio-labeled bacteria (3H-labeled *S. sanguinis*, 14C-labeled *S. mutans*, 3H-labeled *S. sanguinis* mixed with 14C-labeled *S. mutans*) were washed 3 times with PBS. Finally, the cells were adjusted with PBS to a concentration of 10⁹ CFU/mL.

Each resin block (n=5 per group) was immersed in 10 mL of sterilized whole saliva at 37°C for 24 h and washed in distilled water. Then quantitative adherence of the radio-labeled test bacteria was determined. Each saliva-coated resin block was suspended in a test tube with 2 mL of the labeled bacteria at 37°C for 2 h. For removal of the non-adhering bacteria, the resin blocks were withdrawn from the test tubes and immediately washed 3 times with PBS. The labeled bacteria that had adhered to the resin blocks were collected by using an automatic sample combustion system (ASWC-113, Aloka, Tokyo, Japan), and the radioactivity was quantified with a liquid scintillation counter (LSC-903, Aloka).

The values obtained were expressed as disintegrations per min (dpm) and used as an index of the total number of bacteria. Mean and SD were calculated. All numerical data were statistically analyzed.
by using Student’s t-test ($p<0.05$).

**Scanning electron microscope (SEM) observation of adherent bacteria**

The saliva-coated resin blocks used for the adherence test were pre-fixed with 2% glutaraldehyde at 4°C for 2 h, washed twice in 0.1 M sodium cacodylate buffer at pH 7.4, fixed with 1% osmium tetroxide at 4°C for 1 h, and washed twice in the 0.1 M sodium cacodylate buffer at pH 7.4. Finally, the specimens were dehydrated with ethanol and isoamyl acetate and dried with CO₂ by critical point drying. The prepared specimens were placed on aluminum stubs with conductive tape, coated with osmium (HPC-IS, Vacuum Device, Ibaraki, Japan) for 10 s, and observed under an SEM (S-4500, Hitachi, Tokyo, Japan) with secondary electron signals.

**RESULTS**

**Amounts of B, Al, Si, Sr, and F absorbed into salivary coat covering surfaces of S-PRG resin blocks**

The amounts of B, Al, Si, Sr, and F in the whole saliva and SDS solution, and those in the salivary coat covering the surfaces of the resin blocks, are summarized in the Table 2. During the test period, the amounts of B, Al, Si, Sr, and F absorbed into the salivary coat covering the surfaces of unfilled resin blocks, shown as the mean were 18.0, 13.2, 99.6, 1.3 and 90.0 ppb, respectively; whereas the respective values for the salivary coat covering the surfaces of the S-PRG resin blocks were 4,227.4, 722.0, 532.9, 10,857.5 and 691.0. Thus the salivary coat covering the surfaces of the S-PRG resin showed significantly greater amounts of absorbed B, Al, Si, Sr, and F.

**Adherence of S. sanguinis and S. mutans to saliva-coated S-PRG resin blocks**

Student’s t-test revealed that the number of bacteria in the retained dental plaque was influenced by the material and bacteria. The total number of *S. mutans* that adhered to the S-PRG resin soaked in saliva was significantly ($p<0.05$) lower than that of the bacteria that adhered to the unfilled resin soaked in saliva. However, in the case of *S. sanguinis*, no significant difference in adherence could be recognized between saliva-coated S-PRG resin and unfilled resin.

Figure 1 shows the total numbers of *S. sanguinis* and *S. mutans* that adhered to the saliva-coated S-PRG resin and unfilled resin. The adhesion of *S. mutans* to the saliva-coated S-PRG resin was reduced compared with that of *S. sanguinis*.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Amounts of 5 elements in whole saliva (or SDS solution) and absorbed into the salivary coat covering the surfaces of S-PRG resin and unfilled resin blocks (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
</tr>
<tr>
<td>Whole saliva</td>
<td>12.8(0.5)</td>
</tr>
<tr>
<td>SDS solution</td>
<td>0.3(0.1)</td>
</tr>
<tr>
<td>Saliva-coated unfilled resin</td>
<td>18.0(0.2)</td>
</tr>
<tr>
<td>Saliva-coated S-PRG resin</td>
<td>4,227.4(14.2)*</td>
</tr>
</tbody>
</table>

*Significant difference at $p<0.05$ (the comparison of Saliva-coated unfilled resin and Saliva-coated S-PRG resin), $n=5$, Mean (SD)

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**Fig. 1** Total numbers of ³H-labeled *S. sanguinis* (a) and ¹⁴C-labeled *S. mutans* (b) that adhered to the saliva-coated S-PRG resin and unfilled resin blocks (*Significant difference at $p<0.05$).
with that of *S. mutans* to the unfilled resin. In the case of the adherence of the mixture of the 2 strains to the saliva-coated S-PRG resin and unfilled resin, the total adherence of *S. mutans* to the saliva-coated S-PRG resin was reduced compared with that to the unfilled resin, too, as shown in Fig. 2. Moreover, the total counts of *S. mutans* adherent to the saliva-coated S-PRG resin and unfilled resins were decreased compared with those for the *S. mutans* only.

Figure 3 shows SEM photographs of *S. sanguinis*
and *S. mutans* that adhered to the saliva-coated S-PRG resin and unfilled resin and Fig. 4 shows the results when *S. sanguinis* was mixed with *S. mutans*. It was observed by an SEM that the number of *S. mutans* that adhered to the surface of the saliva-coated S-PRG resin was lower than that in the case of the unfilled resin. Co-adherence of *S. sanguinis* and *S. mutans* was found on the surface of the unfilled resin.

**DISCUSSION**

There are various types of resin composites with different chemical compositions that may influence the adherence of oral streptococci to the supporting structure of dental plaque. The ability of dental materials to inhibit the recurrence of caries is an important clinical property. In the process of plaque formation on solid substrate surfaces including teeth and restorative materials, the initial adhesion of the early colonizers to the surface is a very important step. The surface characteristics of the human salivary pellicle formed on resin composite surfaces were presently able to be shown to be influenced by the composition of the resin composite, and might, in turn, have affected the adherence of bacteria to saliva-coated resin composites. Thus, specific interactions between salivary components and bacteria were also involved in the adherence process. Elucidation of the surface components of saliva-coated resin composite may be necessary in order to explore the basis of the resistance of resin composites to bacterial adherence. *S. mitis*, *S. sanguinis*, and *S. oralis* are predominant among the bacterial flora during early plaque formation. Moreover, studies carried out in vivo and in vitro have identified *S. mutans* as one of the most important microorganisms in the etiology of dental caries; and this bacterium has been chosen as a representative oral bacterium, as it has been also found in early plaque. Initial microbial adhesion is important, as it has been found to influence later plaque formation decisively by means of co-adherence and co-aggregation. Co-adhesion is the least studied of all microbial adhesive interactions, and only a few methods to measure it have been described. Hence, it is necessary to investigate the effects of the release of different inorganic elements contained in the S-PRG resin on the co-adherence of *S. sanguinis* and *S. mutans*. In this in vitro study, quantitative adherence of radio-labeled test bacteria to the resin blocks was determined that the saliva-coated S-PRG resin reduced the adherence of *S. mutans* to this resin. Moreover, SEM photographs of bacterial adherence were presented in this report to visualize the topographical differences more clearly on the saliva-coated S-PRG resin and unfilled resin with *S. sanguinis* and *S. mutans*. The S-PRG resin exhibited inhibiting effects on the growth of *S. mutans*. It might also prevent the co-adherence between *S. mutans* and *S. sanguinis*.

Considerable evidence exists for the concept that the unique chemical and physical properties of S-PRG resin results in the formation of a pellicle distinct for that surface. The reduced adhesion of *S. mutans* to the salivary-coated S-PRG resin in these experiments may have two possible explanations. One possibility is that the physico-chemical surface properties of the S-PRG resin result in the formation of a salivary pellicle that inhibits *S. mutans* growth due to the abundance of BO$_3^{3-}$, Al$^{3+}$, SiO$_3^{2-}$, Sr$^{2+}$ and F$^{-}$ ions in the pellicle. We think that the pellicle composition may vary between different restorative materials; and, consequently, different pellicle components such as B and Al would act as binding receptors for bacterial cells. Some dental restorations release metallic or fluoride ions into the environment with a possible influence on the vitality of the adherent bacteria. Glass ionomer cements (GICs), which are widely used as a restorative material, contain a high percentage of F. It was reported that the population of *S. mutans* on the surface of GIC fillings is lower than that on composite resin fillings. Also, the release of fluoride from GICs can prevent caries progression by interfering with the growth or metabolism of the remaining cariogenic bacteria. Fluoride penetration into dental plaque is an important subject, since even limited fluoride penetration may...
serve to inhibit the growth of plaque bacteria. Fluoride is known to inhibit the biosynthetic metabolism of bacteria, but these antimicrobial effects in caries prevention are often regarded as minor compared with the direct interactions of fluoride with the hard tissue during caries development. This inhibition is believed to contribute to the inhibition of the development of secondary caries. Materials with some ions with cariostatic properties can reduce the development of caries through inhibition of bacterial activity. Boron and strontium ions inhibit the growth of caries-origin bacteria. It has already been discussed that other components simultaneously released from ionomeric-based (e.g., aluminum) may be involved in the antibacterial activity of the materials. Moreover, Nakajo et al. reported that ions of silica, aluminum and fluoride released from glass ionomer cements inhibited the fall in pH and acid production as well as streptococcal growth. Thus, the capacity of restorative materials to release such agents as well as the ability of their incorporation in the adjacent dental structure should be considered in the case of tooth protection against secondary caries.

A second possibility is that pellicle components from human saliva, such as lysozyme, cystatin and lactoferrin, may have inhibited the adhesion of these bacteria to the salivary-coated S-PRG resin. These enzymes (lysozyme, cystatin and lactoferrin) may specifically adhere to S-PRG resin. This reduction in the adhesion of the bacteria may have been the result of an inhibitory effect of specific salivary components such as lysozyme and cystatin. It has been reported that salivary lysozyme and salivary lactoferrin are incorporated into salivary pellicles on enamel and dental restorative surfaces in vitro. Both lysozyme and lactoferrin are known to inhibit the adherence of oral bacteria to tooth surfaces. Further studies are necessary to elucidate this aspect more thoroughly by means of ellipsometry to correlate bacterial adhesion with the absorption of saliva constituents. That is, direct characterization of salivary components of the salivary coat on S-PRG resins should be made.

CONCLUSION

This in vitro study was conducted to determine the amounts of ions (BO$_3^{3-}$, Al$^{3+}$, SiO$_3^{2-}$, Sr$^{2+}$ and F$^-$) absorbed into the salivary coat covering the surfaces of S-PRG resin blocks and to assess the adherence of Streptococcus sanguinis and Streptococcus mutans to salivary-coated S-PRG resin blocks. The results revealed that the salivary-coated S-PRG resin showed significantly greater amounts of absorbed B, Al, Si, Sr and F than the salivacoated unfilled resin. It was of particular significance that the salivary coating of S-PRG resin reduced the adherence of S. mutans to this resin. This reduced adhesion of S. mutans seems to be related to the negative effect of the release of different active ingredients (BO$_3^{3-}$, Al$^{3+}$, SiO$_3^{2-}$, Sr$^{2+}$ and F$^-$) from the S-PRG resin.

ACKNOWLEDGMENTS

The authors express their appreciation to SHOFU INC. for providing the materials used in this study.

REFERENCES