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ORIGINAL ARTICLE

Effects of surface reaction-type pre-reacted glass ionomer on oral biofilm formation of *Streptococcus gordonii*

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Abstract *Streptococcus gordonii*, a bacterium involved in the initial colonization of tooth surfaces, contributes to dental biofilm formation and is an important cause of infective endocarditis. This study aimed to investigate the influence of surface reaction-type pre-reacted glass ionomer (S-PRG) filler on oral bacterial growth and aggregation of *S. gordonii*. The effect of various concentrations of S-PRG eluate on the growth and the biofilm formation of *S. gordonii* and other oral microorganisms (*Streptococcus mutans*, *Streptococcus oralis*, *Lactobacillus acidophilus*, and *Candida albicans*) was assessed. In addition, the effect of S-PRG eluate on coaggregation of *S. gordonii* with both *S. oralis* and *Fusobacterium nucleatum* was assessed. The effect of S-PRG eluate treatment on autoaggregation of *S. gordonii* was also evaluated. Our results indicate that S-PRG eluate treatment reduced both for the growth and for biofilm of all organisms in a dose-dependent manner. Coaggregation of *S. gordonii* with both *S. oralis* and *F. nucleatum* was inhibited by S-PRG eluate, whereas autoaggregation of *S. gordonii* increased at certain concentrations of S-PRG eluate. These results indicate that the S-PRG filler possesses antimicrobial activity that is mediated by inhibiting growth and biofilm of oral microorganisms, and by suppressing coaggregation of *S. gordonii*. In addition, these findings indicate that coaggregation of *S. gordonii* with other bacteria is inhibited by increased autoaggregation of *S. gordonii*.

Introduction

Streptococcus gordonii and other closely related species are predominant bacteria in the human oral cavity and are known to colonize tooth surfaces by forming biofilms, which are commonly referred to as "dental plaque" [1]. *S. gordonii* aggregates with other oral bacteria, including other streptococci [2], actinomycetes [3, 4], and *Porphyromonas gingivalis* [5–7] to form biofilms. Bacterial coaggregation, which is defined as cell-to-cell adherence of different bacterial species or strains, is an integral part of plaque formation [8]. Although the cariogenicity of *S. gordonii* is poorly understood, the cariogenicity of *S. mutans* has been characterized by using specific pathogen-free rats [9]. In addition to its ability to cause oral infectious diseases, *S. gordonii* is known to colonize damaged heart valves and is most frequently identified as a primary etiological agent of infective endocarditis [10–12].

For preventing diseases caused by oral microorganisms, it is useful to reduce bacterial growth and to suppress oral biofilm formation. In addition to mechanical methods of maintaining oral hygiene, various other strategies such as the application of antibacterial dental materials have been developed to impede the buildup of bacteria on dental surfaces [13–15]. The antibacterial activity of composite resin containing surface reaction-type pre-reacted glass ionomer (S-PRG) filler was recently reported [16]. With pre-reacted glass ionomer (PRG) technology, a glass

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ionomer phase is formed on glass particles through the aqueous reaction of fluoroaluminosilicate glass with polycarboxylic acid. The S-PRG filler can be recharged with fluoride [17–20]. Various materials such as composite resin, denture base resin, pit and fissure sealants, root canal sealer, and one-step adhesive incorporate S-PRG filler [20–25].

S-PRG filler releases several types of ions (F^-, Sr^{2+}, Al^{3+}, BO_3^{3-}, Na^+, and SiO_3^{2-}) in distilled water and in lactic acid solution [17, 26], and composite resins with S-PRG filler exhibit antibacterial activity through release of metal ions [16]. Yoneda et al. [27] showed that S-PRG eluate inhibits the protease and gelatinase activities of *P. gingivalis* and suppresses coaggregation between *P. gingivalis* and *Fusobacterium nucleatum*. Another study showed that S-PRG eluate inhibits biofilm formation and disrupts mature biofilms [28]. However, the antibacterial activity of S-PRG filler has not been thoroughly assessed.

The purpose of this in vitro study was to evaluate the antimicrobial activity of S-PRG filler against the growth of *S. gordonii* and other oral microorganisms. In addition, the inhibitory activity of the filler against *S. gordonii* autoaggregation and coaggregation with other oral bacteria was also studied.

Materials and methods

Preparation of S-PRG eluate

S-PRG eluate was prepared as described previously under conditions that produce the highest concentration of each ion [26–28]. Briefly, S-PRG filler (Shofu Inc., Kyoto, Japan) was mixed with an equal amount of distilled water and was shaken gently at room temperature for 24 h. The solution was centrifuged to remove filler material, and the supernatant was filter-sterilized (pore size 0.22 μm) to remove residual insoluble materials and other microbial contaminants. The obtained clear solution was used as S-PRG eluate.

Elemental analysis of the S-PRG eluate was performed using inductively coupled plasma atomic emission spectroscopy (ICP-AES; Instrument name, ICPS-8000; Shimadzu Co., Kyoto, Japan), after preparing calibration curves corresponding to each element (standard solutions of Na, Sr, B, Al, Si, and S were used; the standard curve for each element was generated using the following concentrations: 0, 0.5, 5.0, and 20.0 ppm). In addition, the fluoride ion concentration of the S-PRG eluate was measured. Total ionic strength adjustment buffer (0.5 mL; TISAB III; Thermo Fisher Scientific Inc., Waltham, MA) was added to the S-PRG eluate, and a combination fluoride electrode (Orion 9609BN; Thermo Scientific Inc.) that was

connected to a fluoride ion meter (720A; Thermo Scientific Inc.) was inserted into the sample solution to measure the released fluoride. The pH values were measured using a pH meter (pH METER F-22; Horiba Ltd., Kyoto, Japan). Before commencing pH measurement, pH electrodes were calibrated with standard solutions at pH 6.86 and pH 4.01 (Horiba Ltd.).

Bacterial growth assessment

The effect of S-PRG eluate treatment on the growth of the following organisms was assessed: *S. gordonii* DL1; *S. mutans* MT8148 and *Lactobacillus acidophilus* NDU121 (known as causative agents of tooth decay [29, 30]); *S. oralis* 34 (an early colonizer of the oral cavity and a causative agent of infective endocarditis [31, 32]); and *Candida albicans* NDU221 (causative agent of candidiasis [33, 34]). The two NDU strains were clinically isolated from human oral mucosa under the approval of the Ethics Committee for the Nippon Dental University School of Life Dentistry at Tokyo (approval number NDU-T2013-03) and checked to possess typical biological properties. Organisms were cultured in brain heart infusion (BHI) broth (Becton, Dickinson and Company, Sparks, MD) at 37 °C for 18 h.

Test cultures were grown under three test conditions to evaluate the effects of S-PRG eluate on bacterial growth: $1 \times$ BHI (control, 4 mL of $1 \times$ BHI); $1 \times$ BHI with 20 % S-PRG eluate (2 mL of $2 \times$ BHI, 0.8 mL of S-PRG eluate, and 1.2 mL of sterilized distilled water); and $1 \times$ BHI with 50 % S-PRG eluate (2 mL of $2 \times$ BHI and 2 mL of S-PRG eluate). The bacterial inoculum was obtained by growing the cultures at 37 °C for 18 h. The test cultures were set-up by inoculating the inoculum bacteria in BHI to get a final bacterial optical density (OD) of 0.01 (A_{620nm}) with a final culture volume of 4 mL. Bacterial cell growth in static culture at 37 °C was automatically recorded at A_{650nm} using a TVS062CA Bio-photorecorder (Advantec, Tokyo, Japan). Three independent experiments were performed for each assay.

Biofilm assays

Biofilm formation was assessed as described previously [35] with some modifications. The biofilm medium (BM) contained 58 mM K_2HPO_4, 15 mM KH_2PO_4, 10 mM $(NH_4)_2SO_4$, 35 mM NaCl, 0.8 % glucose, 0.2 % casamino acids, and 0.05 mM $MnCl_2 \cdot 4H_2O$ and was supplemented with filter-sterilized vitamins (0.04 mM nicotinic acid, 0.1 mM pyridoxine HCl, 0.01 mM pantothenic acid, 1 mM riboflavin, 0.3 mM thiamin HCl, and 0.05 mM D-biotin), amino acids (4 mM L-glutamic acid, 1 mM L-arginine HCl, 1.3 mM L-cysteine HCl, and 0.1 mM L-tryptophan),

and 2 mM MgSO$_2$·7H$_2$O. Round-bottomed polystyrene microtiter plates (Falcon®, NY, USA) containing 200 mL of test culture per well were inoculated with an 18-h bacterial culture (final $A_{595nm} = 0.01$) under four test conditions: 1 × BM (control); 1 × BM with 5 % S-PRG eluate; 1 × BM with 20 % S-PRG eluate; and 1 × BM with 50 % S-PRG eluate. Six independent experiments were performed for each assay. After 16 h of incubation at 37 °C under anaerobic conditions with agitation, 25 μL of 0.2 % crystal violet (CV) solution was added to each well. After 15 min, wells were rinsed two times with 200 μL of distilled water and dried. CV-stained biofilm on the bottom of the well was photographed using a scanner, and the images were analyzed using ImageJ version 1.48 software. Statistical differences in the means of optical values were evaluated by performing an unpaired t test. Differences were considered to be significant at $P < 0.001$.

Coaggregation assay of S. gordonii and other bacteria

Coaggregation assay was conducted according to the protocols described by Cisar et al. [36] and Kolenbrander et al. [37]. The assay was performed using two microbial combinations: (a) S. gordonii DL1 and S. oralis 34, and (b) S. gordonii DL1 and F. nucleatum ATCC25586. A previous study demonstrated strong coaggregation reactions between S. gordonii DL1 and F. nucleatum ATCC25586 [38, 39]. In this study, streptococci were cultured as described above, and F. nucleatum was incubated anaerobically in BHI broth at 37 °C for 2–3 days. After incubation, cultures were centrifuged for 15 min at $800 \times g$. Bacterial cells were washed twice in coaggregation buffer [CB; 10 mM Tris, 0.15 M NaCl, 0.1 mM CaCl$_2$, 0.1 mM MgCl$_2$ (pH 7.8)], centrifuged for 15 min at $800 \times g$, and then resuspended in CB to obtain an optical density of 2.0 at 620 nm. Strains to be examined for coaggregation were combined with an equal volume of a test solution (CB as a control) in a total volume of 1.0 mL. To evaluate the effects of S-PRG eluate on coaggregation, the eluate was added to the mixture at final concentrations of 5, 25, and 50 %. After mixing, suspensions were kept at room temperature for 10 min, and the degree of coaggregation in the mixed suspensions was scored using a visual scoring system (from "−" to "4+") [36, 40] as follows: "−" indicates no change in turbidity and no evidence of coaggregates in the mixed suspensions; "1+" indicates turbid supernatant with finely dispersed coaggregates; "2+" indicates definite coaggregates that do not precipitate immediately; "3+" indicates slightly turbid supernatant with formation of large precipitating coaggregates; and "4+" indicates clear supernatant and large

coaggregates that precipitate immediately. All assays were repeated at least three times. Representative results are shown with an indication of reproducibility.

Autoaggregation assay of S. gordonii

Measurement of the autoaggregation percentage of S. gordonii was evaluated as described below. S. gordonii was cultured for 18–24 h and then centrifuged for 15 min at $800 \times g$. The pellet was washed twice in CB and resuspended in CB to obtain an optical density of 2.0 at 620 nm. The bacterial suspension (100 μL) was mixed with an equal volume of the S-PRG eluate, which was previously diluted to various concentrations (final concentrations 0–20 %). The bacteria and eluate mixture (200 μL) was incubated in each well of a 96-well microtiter plate for 30 s. The mixture was then centrifuged for 2 min at $10 \times g$ (Tabletop Centrifuge KN-70; Kubota Corp., Tokyo, Japan) and supernatant (50 μL) was gently transferred to a new 96-well flat-bottomed microtiter plate. Optical density of the solution at 650 nm was measured using the SpectraMax Plus (MDS Analytical Technologies, Sunnyvale, CA). Three independent experiments were performed for each assay. Degree of autoaggregation was calculated as a percentage using the equation:

$$\text{Autoaggregation} (\%) = (1 - OD_A/OD_B) \times 100,$$

where OD_A was the optical density of the solution with 0 to 20 % S-PRG eluate, and OD_B was the optical density of the control solution.

Results

Ion release and pH levels

The results of elemental analysis of S-PRG eluate were as follows: Al, 20.8 ppm; B, 1683.8 ppm; Na, 554.2 ppm; Si, 10.3 ppm; Sr, 149.6 ppm; and F, 137.0 ppm. pH of the eluate was 7.7; all experiments were performed using this single batch. No pH changes or precipitation was observed after the addition of eluate to the reaction mixtures.

S-PRG eluate reduces bacterial growth

Bacterial cell growth rates of each organism after treatment with S-PRG eluate are shown in Fig. 1. The growth rate of the untreated control culture was considerably higher than those of cultures treated with S-PRG eluate. In addition, the growth inhibition occurred in a dose-dependent manner. Remarkably, growth of C. albicans was completely inhibited in culture containing S-PRG eluate (Fig. 1e).
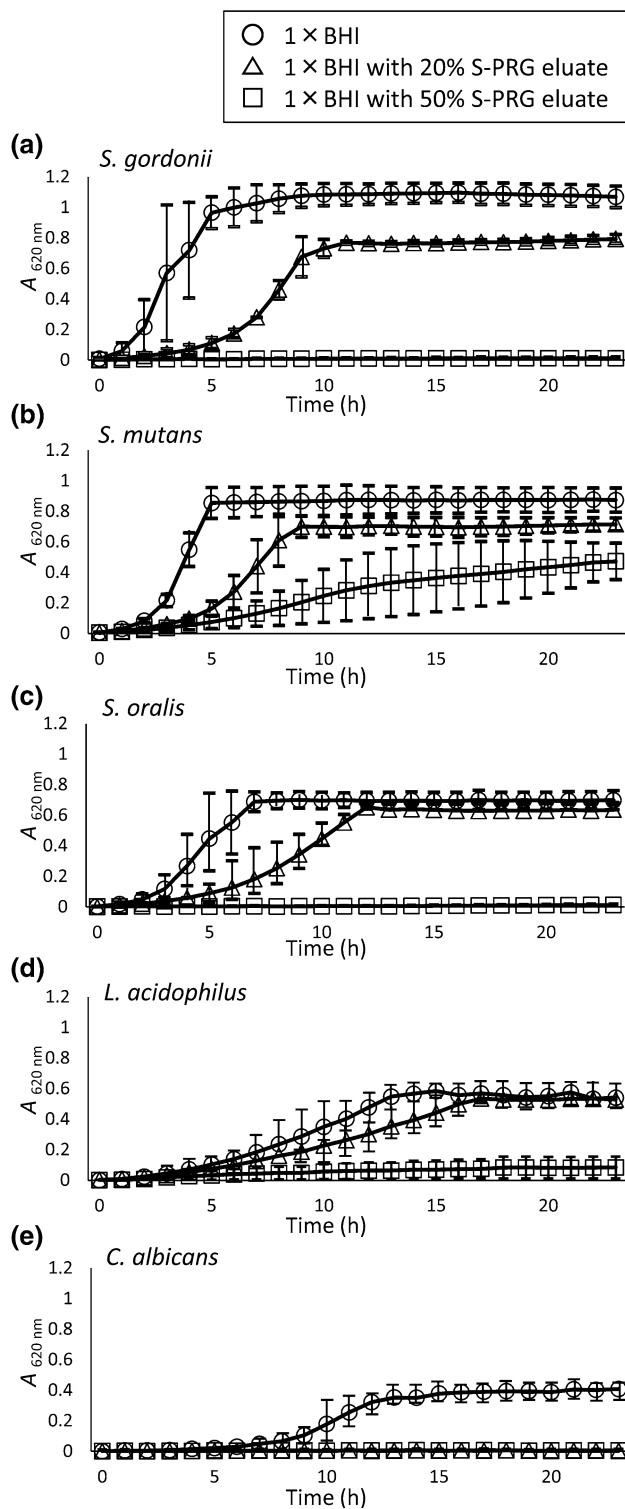
Fig. 1 Effect of S-PRG eluate treatment on the growth rates of various oral bacteria. *Open circles*, *open triangles*, and *open squares* indicate 1 × BHI (control), 1 × BHI with 20 % S-PRG eluate, and 1 × BHI with 50 % S-PRG eluate, respectively. The assays were performed using **a** *Streptococcus gordonii* DL1, **b** *S. mutans* MT8148, **c** *S. oralis* 34, **d** *Lactobacillus acidophilus* NDU121, and **e** *Candida albicans* NDU221. Data represent mean values and the *whiskers* indicate standard deviation (*n* = 3)

S-PRG eluate reduces biofilm formation

The optical values of CV-stained biofilm formation of each streptococcus species after treatment with S-PRG eluate are shown in Fig. 2. The *S. gordonii* biofilm formation on polystyrene surface was significantly reduced in BM broth containing S-PRG eluate (Fig. 2a). For *S. mutans* and *S. oralis*, the optical values of CV-stained biofilms cultured in media containing 25 % S-PRG eluate and 50 % S-PRG eluate were significantly lower than those of the control cultures ($p < 0.001$, Fig. 2b, c). In contrast to that observed with streptococci cultures, no biofilm formation was observed with *L. acidophilus* and *C. albicans* control cultures (Fig. 2d).

Effects of S-PRG eluate on coaggregation

Profiles of coaggregation between *S. gordonii* and *S. oralis* or *F. nucleatum* in the presence or absence of S-PRG eluate are shown in Fig. 3. For the assay using *S. gordonii* and *S. oralis*, coaggregation scores of "2+," "1+," "−," and "−" were obtained after treatment with 0, 5, 25, and 50 % S-PRG eluate, respectively. For the assay using *S. gordonii* and *F. nucleatum*, coaggregation scores of "4+," "4+," "3+," and "3+" were obtained after treatment with 0, 5, 25, and 50 % S-PRG eluate, respectively. The formation of precipitating coaggregates was reduced only by certain concentrations of S-PRG eluate.

Effects of S-PRG eluate on autoaggregation

Degree of autoaggregation of *S. gordonii* in the presence of S-PRG eluate was assessed and is shown in Fig. 4. The degree of autoaggregation was low at S-PRG eluate concentrations of 8 % or lower. However, treatment with eluate concentrations of 10–16 % showed a steep increase in the degree of autoaggregation with increasing concentrations of the eluate. Notably, treatment with 18 % S-PRG eluate resulted in degree of autoaggregation that was higher than 80 %.

Discussion

The elemental analysis of S-PRG eluate showed that it contained considerably high amounts of BO_3^{3-}, and relatively high amounts of F^-, Sr^{2+}, Al^{3+}, Na^+, and SiO_3^{2-}. Although the mechanisms of ion release from S-PRG filler are not completely understood, it is believed that the presence of a glass ionomer phase around the glass core of the filler is related to ion release [19]. Among the ions detected in this study, boron and fluoride ions released from S-PRG filler may possess antibacterial activity [41].
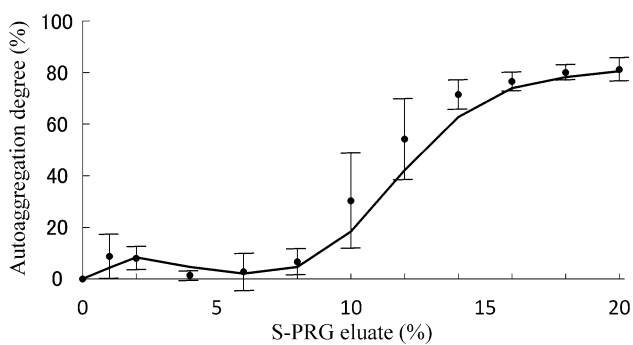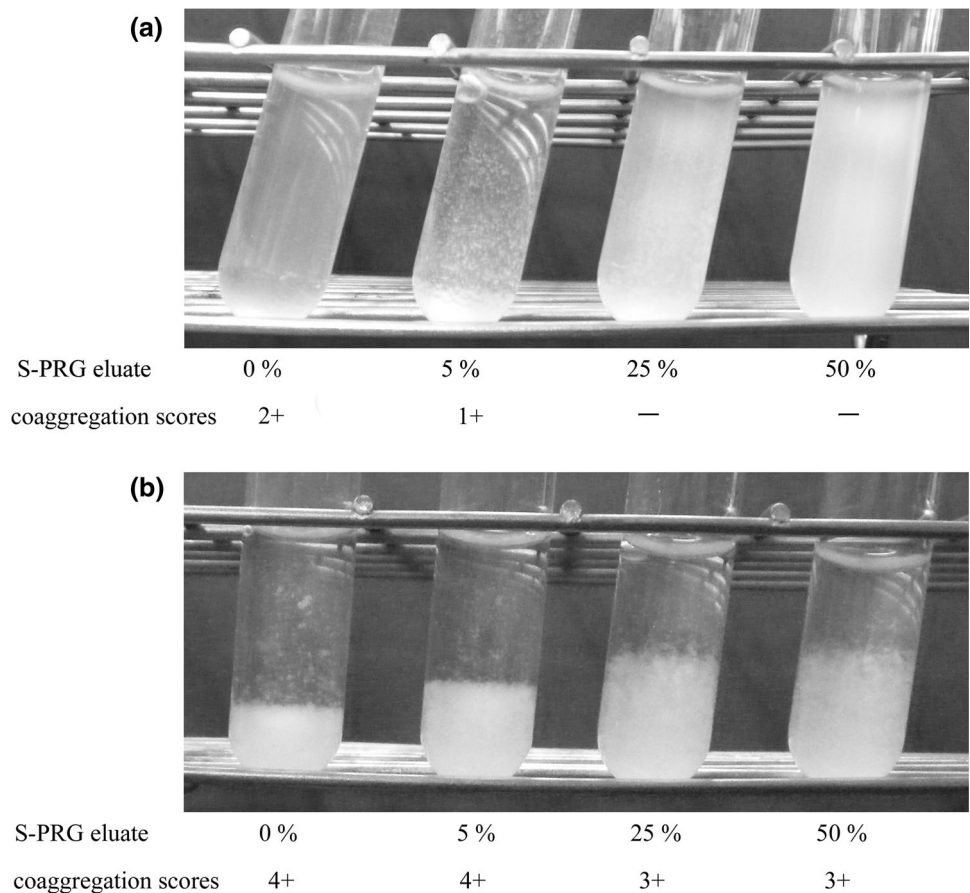
(a) *S. gordonii*

(b) *S. mutans*

(c) *S. oralis*

(d)
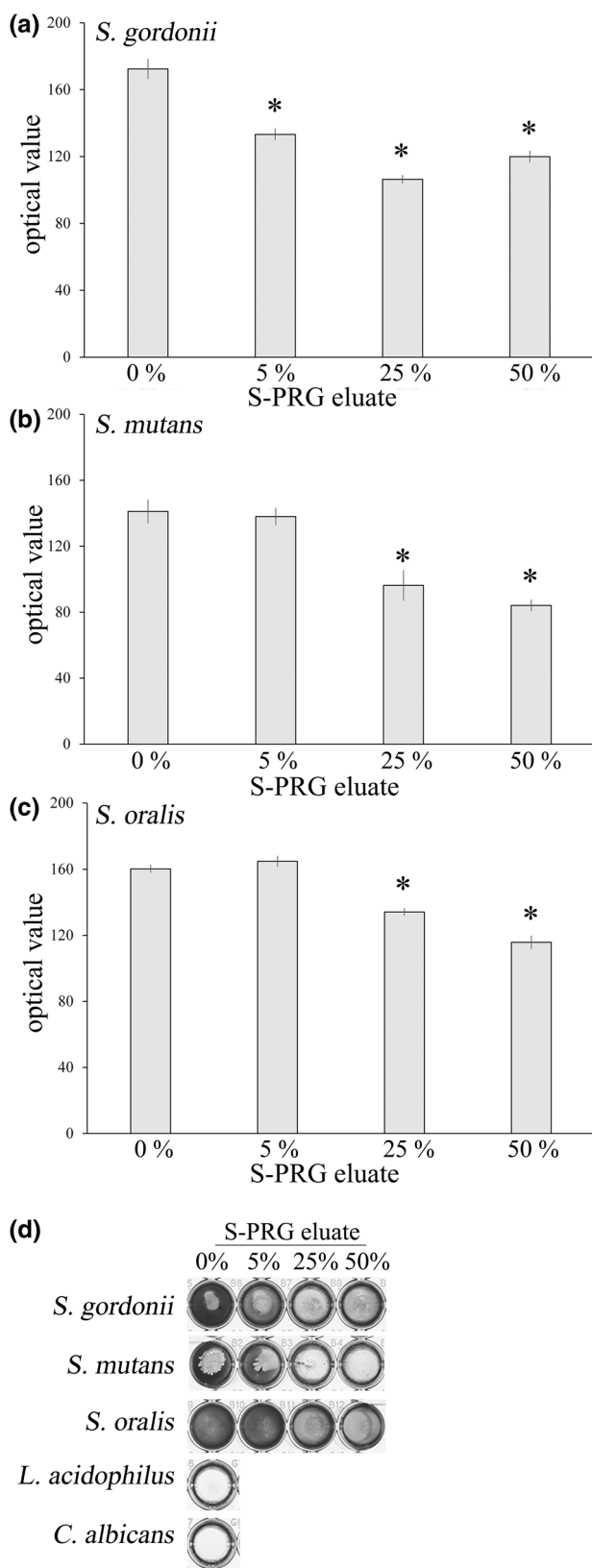
◄**Fig. 2** Effect of S-PRG eluate treatment on biofilm formation of various oral streptococci. Mean ($n = 6$) and SD of the optical values of CV-stained biofilms are indicated. The *asterisk* indicates that the optical values were significantly less than that of the control (0 % S-PRG eluate; $P < 0.001$). The assays were performed using **a** *S. gordonii* DL1, **b** *S. mutans* MT8148, and **c** *S. oralis* 34. Biofilm of each bacterial culture grown in the presence of various concentrations of S-PRG eluate was stained with crystal violet (**d**)

In this study, the bacterial growth rates were lower in the presence of S-PRG eluate and the growth inhibition was dose dependent. In addition, inhibitory effect of S-PRG on the biofilm formation of oral streptococci was observed. It is possible that the ions released from S-PRG filler contribute to the inhibition of bacterial cell growth and biofilm formation. Interestingly, the growth of *C. albicans* was strongly inhibited in the culture containing S-PRG eluate. This indicates that denture base resins containing S-PRG filler could suppress oral candidiasis.

Coaggregation is thought to be important in the development of oral biofilms, as the ability of cells to bind to tooth-associated biofilm affords an opportunity to join the developing microbial community [42]. *S. gordonii* surface proteins, Hsa, and SspA/SspB, are known to adhere to salivary proteins and mediate coaggregation with other bacteria [43–45]. In this study, coaggregation between *S. gordonii* and other bacteria was inhibited depending on the concentration of S-PRG eluate. The formation of precipitating coaggregates was suspected to be inhibited by inactivation of the bacterial cell surface layer protein by the ions released from S-PRG eluate; however, further analysis of these properties needs to be carried out. In addition, a previous study has shown that the *S. gordonii* surface protein Hsa mediates binding of the organism to saliva-coated hydroxyapatite [46]. Thus, the utilization of S-PRG in dental materials could be useful in preventing bacterial biofilm development.

Autoaggregation is defined as the adherence of bacteria belonging to the same strain [47]. In a previous study, *prsA* mutants of *S. mutans* displayed an altered cell wall protein profile that led to increased autoaggregation, reduced resistance to mechanical breakage, and early biofilm formation [48]. Although the autoaggregation of *S. gordonii* has not been reported previously, autoaggregated bacteria may attenuate the increase in coaggregation with other bacteria and may be easily removed from the pellicle by brushing. In the present study, autoaggregation of *S. gordonii* increased depending on the concentration of S-PRG eluate used. Autoaggregation is thought to be influenced by

Fig. 3 Effect of S-PRG eluate on microbial coaggregation. Photographs of coaggregation between **a** *S. gordonii* DL1 and *S. oralis* 34, and **b** *S. gordonii* DL1 and *F. nucleatum* ATCC25586 are shown. The coaggregation score of each S-PRG eluate concentration is indicated below the photograph

S-PRG eluate	0 %	5 %	25 %	50 %
coaggregation scores	2+	1+	—	—

S-PRG eluate	0 %	5 %	25 %	50 %
coaggregation scores	4+	4+	3+	3+

Fig. 4 Effect of S-PRG eluate on autoaggregation of *S. gordonii* DL1. The effect of various concentrations of S-PRG eluate on autoaggregation of *S. gordonii* DL1 is shown in the *graph*. *Solid black circles* represent mean values and the *whiskers* indicate standard deviation (*n* = 3)

positive ions (Sr^{2+}, Al^{3+}, and Na^+) released from S-PRG filler, because bacterial cell surfaces are negatively charged due to the dissociation of carboxyl and phosphate groups under neutral conditions. In our previous studies, we measured the amounts of various ions released from a specimen of sealant containing S-PRG filler (internal diameter, 15 mm; height, 1 mm) into 5 mL distilled water.

The levels of F^-, Sr^{2+}, BO_3^{3-}, Al^{3+}, Na^+, and SiO_3^{2-} ions released were found to be 12.60 ppm, 7.39 ppm, 6.60 ppm, 0.90 ppm, 15.01 ppm, and 1.02 ppm, respectively [17]. As the ions released from resin are in small quantities, the S-PRG filler may have limited antimicrobial efficacy and inclusion of S-PRG filler in toothpastes may have better antimicrobial effectiveness. Additional studies are needed for examining the antimicrobial activity of each ion as well as of the various combinations of ions. In addition, the optimal safe concentrations of these ions should be determined.

In conclusion, the results of our study indicate that the S-PRG filler has inhibitory activity both for microbial growth, biofilm, and aggregation of *S. gordonii*. Thus, dental materials containing S-PRG filler have the ability to inhibit oral biofilm formation.

Compliance with ethical standards

Conflict of interest The S-PRG filler was provided by Shofu Inc. (Kyoto, Japan); however, the sponsor of the study had no role in the

study design, conduct of the study, data collection, data interpretation, or preparation of the report.

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