Inhibition of Biofilm Formation using Newly Developed Coating Materials with Self-cleaning Properties

Ayako OKADA1, Toru NIKAI1DO1, Masaomi IKEDA1, Koichi OKADA2, Junichi YAMAUCHI2, Richard M. FOXTON3, Hideo SAWADA4, Junji TAGAMI5 and Khairul MATIN1,5

1Department of Operative Dentistry, School of Dentistry, Okayama University, Okayama, Japan
2Kuraray Medical Inc., 2045-1, Sakazu, Kurashiki-shi, Okayama 710-8691, Japan
3Division of Conservative Dentistry, King’s College London Dental Institute at Guy’s, King’s College and St. Thomas’ Hospitals, King’s College London, London SE1-9RT, UK
4Department of Frontier Materials Chemistry, Faculty of Science and Technology, Hirosaki University, 3 Bunkyo-cho, Hirosaki, Aomori 036-8561, Japan
5Center of Excellence Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan
Corresponding author, Ayako OKADA; E-mail: hisoppu2003@yahoo.co.jp

The purpose of this study was to evaluate the inhibition of biofilm formation on newly developed coating materials with self-cleaning properties. A series of experimental coating materials containing fluoroalkylated acrylic acid oligomer (FAAO) were applied to composite substrates. The surfaces of the coating materials were analyzed by X-ray photoelectron spectroscopy (XPS) and contact angle measurement. Biofilm formation on the surface was assessed using Streptococcus mutans biofilms inside an oral simulator in vitro. The results indicated that an increase in the concentration of FAAO in the coating materials enhanced surface hydrophilicity and oil-repellency. Biofilm assays demonstrated that the amount of biofilm retained on the coating materials gradually decreased when the concentration of FAAO increased in the materials. It was concluded that the coating materials incorporated with FAAO possessed self-cleaning properties and displayed signs of inhibiting biofilm formation on their surfaces.

Key words: Coating material, Fluoroalkylated acrylic acid oligomer, Biofilm

INTRODUCTION

In recent years, novel surfaces with “self-cleaning” surface properties have generated immense interest in biomaterial research and development. Indeed, if surfaces with controlled wetting properties could be fabricated, then the quest for contamination-free surfaces would become a long-awaited reality.

From the manufacturing perspective of dental materials, several methods have been proposed for the development of restorative materials and adhesive materials with an ability to influence oral biofilm formation. Development of dental materials possessing “self-cleaning” surface properties would bring a dramatic change to the field of clinical dentistry. For example, such surface coating materials on enamel and dentin would definitely save more teeth. For restorative materials, denture base materials, and implants, their longevity would be increased because of protection against bacterial attack. However, to date, a dental material with such a desirable and beneficial property is still not available.

Bacterial species have a strong tendency to colonize surfaces. Biofilm formation is a multifactorial infection process, which is also influenced by the physiochemical properties of the substrate surface. In particular, the wettability property of a dental material exerts its influence on many fronts: contact with intraoral fluids (predominantly saliva), adsorption of salivary proteins, adhesion of bacteria and/or biofilms, and frictional forces exerted by affecting the oral tissues or food particles. Contacting the orthodontic appliance. Further on wettability, it has been reported that the superhydrophobicity and superoleophobicity of surfaces are key properties for the fabrication of self-cleaning surfaces.

In 1990s, Sawada et al. introduced a newly categorized chemical compound with a self-cleaning property, constituting the first generation of current “fluoroalkylated acrylic acid oligomer” (FAAO). A FAAO is generally synthesized by the polymerization of acrylic acid with difluoroalkanoyl peroxides (Fig. 1). The FAAO can reduce the surface tension of both water and organic solvents.

With a view to developing a new coating material with a self-cleaning property for oral use, an experiment was conducted in which FAAO was
incorporated in varying proportions into a photoinitiator containing acrylate monomer. The aim of this study was to evaluate the surface properties and biofilm formation on FAAA-incorporated materials. The experimental hypothesis was that FAAA-incorporated materials have a self-cleaning property and the potential to inhibit biofilm formation on the surface.

MATERIALS AND METHODS

Experimental coating materials

A schematic illustrating the synthesis of fluoroalkylated acrylic acid oligomer (FAAO) is shown in Fig. 1. FAAA was synthesized according to previous studies reported by Sawada et al.\textsuperscript{11,12}. Perfluoro-2,5-dimethyl-3,6-dioxanonanoyl peroxide (5 mmol, 4.9 g) was dissolved in 65 g of a fluorocarbon solvent (a mixed solvent in which the volume ratio of CF\textsubscript{3}CF\textsubscript{2}CH\textsubscript{2}Cl to CF\textsubscript{3}CF\textsubscript{2}CF\textsubscript{2}Cl was 1:1, manufactured by ASahi GLASS Co., Ltd. under the product number AK-225), followed by addition of acrylic acid (49 mmol, 3.6 g). The resulting mixture was reacted at 45°C in an atmosphere of nitrogen for five hours. After the reaction had terminated, the resulting white powder was collected by filtration. The crude product was washed with hexane for purification and then dried at 50°C in vacuo for two days to give a bis(perfluoro-2, 5-dimethyl-3, 6-dioxanonanylated) acrylic acid oligomer (4.7 g).

This oligomer showed the following spectral characteristics: IR v/cm\textsuperscript{-1} 3200 (OH), 1720 (C=O), 1335 (CF\textsubscript{3}), 1240 (CF\textsubscript{2}); 'H-NMR (CD\textsubscript{3}OD) δ 1.35-2.19 (CH\textsubscript{2}-), 2.21-2.72 (CH); average molecular mass (Mm) = 4250 (determined by gel permeation chromatography, eluted with tetrahydrofurran).

A series of experimental coating materials incorporating FAAA were then prepared. Table 1 lists the compositions of these experimental coating materials. The coating materials contained 0, 0.1, 0.49, and 0.98 wt\% of FAAA, difluoroalkanoyl peroxide, MMA, ethanol, and photoinitiator, and were coded as F-0, F-I, F-II, and F-III respectively. The experimental materials were kept in dark glass bottles at 4°C before use.

Specimen preparation

An indirect composite (Estenia C&B, E1 Shade, Kuraray Medical, Tokyo, Japan) was used to prepare square-shaped composite blocks. The specimens were polished with 800-grit SiC paper under running water. The top surfaces of the composite blocks were silanized by applying a commercially available silane coupling agent (Clearfil Ceramic Primer, Kuraray Medical) and heat-curing at 110°C for 15 minutes in an oven (KL-100, J. Morita Corp., Osaka, Japan). Then, 5 µl of each experimental coating material was applied to the composite surfaces and spun at 10,000 rpm (10,840 g) for 10 seconds to ensure that the surfaces were thin and smooth. Following this, all specimens were immediately kept in darkness for 20 minutes to allow evaporation of ethanol from the coating materials, then light-cured for three minutes with a visible light curing unit (Alpha-Light II, J. Morita Corp.). The specimens were stored overnight in the presence of UV light to render the surfaces free from contamination.

Surface chemical analysis by XPS

For elemental analysis of the surfaces of the coating materials, square-shaped specimens (4×4×1.5 mm\textsuperscript{3}) were prepared in the same manner as described above. X-ray photoelectron spectroscopy (XPS) measurements were performed on a Kratos AXIS-HS spectrometer (Shimadzu, Kyoto, Japan) with an Mg K\textalpha\ X-ray source (1253.6 eV photons). Test specimens were mounted on standard sample studs.
by means of double-sided adhesive tape. A takeoff angle of 90° was used in all XPS runs. The X-ray source was operated at 15 kV and 10 mA, and the measurement area was confined to 600 µm × 800 µm. Pressure in the analysis chamber was maintained at 3×10⁻⁶ Pa or lower during measurements. To compensate for surface charging effects, all binding energies were referenced to the C1s neutral carbon peak at 285 eV.

Contact angle measurement
Square-shaped specimens (20×20×1.5 mm³) were prepared as indicated above and spin-coated with 125 µl of the experimental coating materials on each sample. The contact angles were measured using a contact angle device (FTA125, First Ten Ångstroms, Portsmouth, Virginia, USA) at 25°C. For surface analysis of the hydrophilic characteristics, 3.5 µl of deionized water (Milli-Q Plus system, Japan Millipore, Tokyo, Japan) was dropped on the surface, and then video images were taken. To evaluate oil repellency, 3.5 µl of hexadecane (Wako Pure Chemical Industries Ltd., Wako, Japan) was applied. Video images were automatically inputted to an attached computer in which the contact angles were measured using an image analysis program (FTA32 video, First Ten Ångstroms). Contact angles were measured every second for a period of 20 seconds at 25°C.

Artificial biofilm formation on the specimens and adherence measurement assay
To evaluate the behavior of the coating materials relative to biofilm formation, a procedure similar to that previously reported was employed. In brief, a suspension of *Streptococcus mutans* MT8148 (S. mutans) in phosphate buffered saline (PBS) at OD₅₆₀=2 (approximately 2×10⁹ colony-forming units/ml) was prepared from a 16-hour fresh culture in Brain Heart Infusion (BHI, Becton Dickinson, Sparks, MD, USA) broth. After washing three times with PBS, the suspension was stored at 4°C with gentle stirring. For the growth of the biofilms, a solution of Heart Infusion (HI, Becton Dickinson) broth with sucrose (1% final concentration) was used.

Specimens (4×4×1.5 mm³) were prepared after spin-coating each substrate with 5 µl of FAAO coating material as described above. Four specimens from each group were placed on a Teflon holder of an oral biofilm reactor (OBR) by using red utility wax (GC, Tokyo, Japan). This was done so that only the experimental surface was exposed for biofilm formation. Pooled sterile saliva was poured on the specimen surfaces and incubated for 30 minutes to obtain a coating of salivary pellicle. Artificial *S. mutans* biofilms were then grown on specimens inside two identical water jacket-encircled chambers of the OBR (Fig. 2). After 20 hours, each specimen was subjected to shaking with a TissueLyser (Retsch Gmbh & Co., Haan, Germany) at 30 Hz for 150 seconds in chilled PBS.

After shaking, the retained biofilms were measured by separating the bacterial cells and water-insoluble glucan (WIG) according to a method previously described. Each specimen was transferred carefully from the PBS to 1 ml of 0.5 mol/l sodium hydroxide solution, incubated for 15 minutes, vortexed, and centrifuged at 5,000 rpm for 10 minutes to separate the WIG and bacterial cells embedded in the biofilms. Each bacterial pellet was resuspended in 1 ml of PBS and 100 µl of each bacterial cell suspension, and then transferred to separate wells of a 96-well flat-bottom microplate. Turbidimetric analysis (OD₅₆₀nm) was performed with a Biotrak II Plate reader (Biochrom, Cambridge, UK) to quantify the bacteria. Dissolved WIG amount was measured by the phenol-H₂SO₄ method, and absorbance at 492 nm was determined with a Biotrak II Plate reader. To calculate the WIG amount (µg/ml), 500 µl of WIG solution from each sample was dissolved in phenol-H₂SO₄, and 200 µl of each of the resulting solutions was subjected to analysis.

Statistical analysis
All numerical data were analyzed using the
Statistical Package for Medical Science (SPSS Ver. 11 for Windows) for statistical procedures. The number of specimens for contact angle analysis was five for each group. Regression analysis was performed by non-linear regression using the concentration of FAAO incorporated into the coating materials relative to the contact angle. For biofilm assays, data for the amounts of bacteria/mm² and WIG/mm² were analyzed by Kruskal-Wallis variance analysis test. The biofilm experiments were repeated three times under the same conditions to ensure reproducibility (n=4 in each group).

RESULTS

Surface chemical analysis by XPS
The presence of fluorine along the solid-air interface of the coating material was detected by XPS (Fig. 3). Fluorine peaks at 689 eV could be detected in the F-I, F-II, and F-III specimens in the XPS spectra. No peak was detected in the case of F-0 since it contained no fluorine atom or Rf, and the peak for F-I was smaller than the peaks for F-II and F-III. However, the intensity of the fluorine atom was nearly the same for F-II and F-III.

Contact angle measurement
Results of the water (Milli-Q) and oil (hexadecane) contact angles on the surfaces of the coating materials are summarized in Fig. 4. In the case of the water contact angle, there was a gradual reduction in the contact angle when the FAAO concentration in the coating materials increased. Water contact angle correlated with the concentration of FAAO in the coating material ($r^2$=0.917, p<0.05). The opposite results were obtained for hexadecane, whereby there was a gradual increase in the oil contact angle with increasing concentrations of FAAO incorporated into the specimens with a significant correlation ($r^2$=0.992, p<0.05) in the reverse direction.

Measurement of artificial biofilm formation on the specimens
Regarding biofilm formation, S. mutans biofilms formed on the surfaces of all the specimens after 20 hours of incubation inside the artificial oral simulator. The biofilm remaining on the surface after vigorous shaking was considered as the retained biofilm on the surface. The amounts of retained bacteria and water-insoluble glucan (WIG) are summarized in Table 2. There was a gradual reduction in bacterial accumulation with an increase in the concentration of FAAO in the coating materials, except for F-I. In the case of F-III, the retained biofilm was ranked the lowest among the series of coating materials. With regard to the quantity of WIG, a gradual reduction was also observed when the concentration of FAAO in the coating materials was increased. However, there were no significant differences in the amount of retained biofilm among the series of coating materials (p>0.05).

![Fig. 3 XPS spectra of FAAO-incorporated coating materials.](image-url)
Fig. 4 Scatter diagrams with linear regression (a) and images (b) presenting contact angles of Milli-Q water and hexadecane droplets on the coated specimens.
(a) An inverse relationship between water contact angle and concentration of FAAO was detected, indicating hydrophilic characteristic. However, oil contact angle increase correlated with the concentration of FAAO, showing oil repellency.
(b) Droplet images at the time of contact angle measurement, where: Upper-left: Milli-Q water droplet showing contact angle on F-0, Upper-right: Milli-Q water droplet showing contact angle on F-III, Lower-left: Hexadecane droplet showing contact angle on F-0, Lower-right: Hexadecane droplet showing contact angle on F-III.

Table 2 Amounts of bacteria and water-insoluble glucan (WIG) of the retained biofilms on the coating materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Amounts of bacteria/mm²</th>
<th>WIG/μg/ml/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-0</td>
<td>0.046±0.01</td>
<td>5.98±1.50</td>
</tr>
<tr>
<td>F- I</td>
<td>0.047±0.01</td>
<td>5.23±0.53</td>
</tr>
<tr>
<td>F- II</td>
<td>0.038±0.01</td>
<td>4.59±0.54</td>
</tr>
<tr>
<td>F- III</td>
<td>0.033±0.02</td>
<td>4.59±1.54</td>
</tr>
</tbody>
</table>

Statically no significant differences were detected among the groups (p>0.05).
DISCUSSION

An attempt to develop a new coating material with a self-cleaning surface property was made by incorporating FAAO with a photo-activated acrylic material. Figure 5 explains the theoretical self-cleaning behavior of the FAAO-incorporated material. When water droplets were placed on the surface, the hydrophilic units (-COOH) were attracted toward the water molecules. When oil droplets were placed, the hydrophilic units were pushed flat on the surface allowing fluoroalkyl units (RF) to assume erectile positions—hence making the surface more oil-repellent. Alterations in the positioning of RF and hydrophilic units are referred to as the "flip-flop" characteristic of FAAO-incorporated coating materials.  

The spin coating technique employs a centrifugal force to spread the coating material homogeneously over the entire substrate to form a thin and smooth surface. In particular for XPS analysis, contact angle and surface free energy measurements, specimens with a smooth and uniform surface texture at sub-micrometer level are a prerequisite. Moreover, surface conditions influence biofilm formation on the material surface.

From the XPS spectra results, fluorine was not detected in F-0 which was without FAAO. Conversely, fluorine peaks due to FAAO were detected in F-II and F-III, which were higher than in F-I. However, the intensities of the peaks in F-II and F-III were similar. This result suggested that the presence of aligned FAAO along the solid-air interface was fully saturated in F-II.

The surface characteristics of FAAO-incorporated specimens were investigated by measuring contact angles using water and hexadecane droplets. With increasing concentrations of FAAO, the coating materials tended to display enhanced hydrophilicity and oil repellency. Interestingly, the surface of F-III displayed both superhydrophilicity (contact angle with water was below 30°) and oil repellency (contact angle with hexadecane increased to above 80°). F-III produced differences of nearly 10° in contact angle (both in contact with water and oil) compared to F-II and showed even larger differences when compared

Rf-(CH₂-CH₈)₈-Rf  \[ \Rightarrow \]  Rf

\[ Rf = -CF(CF₃)₅OCF₃CF(CF₃)₃OCF₃, \ m = 0, 1, 2 \]

\[ X = \text{Hydrophilic Unit} \]

Surface condition in response to oil contact  \[ \Rightarrow \]  Surface condition in response to water contact

Fig. 5 Flip-flop model diagram. FAAO was obtained with polyacrylic acid blocked with fluoroalkyl units at both ends. Flip-flop type of switching was anticipated with the incorporation of FAAO. When oil was placed, the hydrophilic units were pushed flat on the surface allowing RF units to assume erectile positions. In contrast, when water came into contact, the hydrophilic units were attracted toward the water molecules and more water molecules were pulled toward the surface.
to F-I. It might be noteworthy that F-III was the only surface that displayed a water contact angle less than 30° (about 21°)—and that is generally categorized as superhydrophilic. This phenomenon could be explained by the unique "flip-flop" behavior of FAAO (shown in Fig. 5) on the material surface. Where, the more hydrophilic units of FAAO were attracted the more water molecules were pulled toward the surface, resulting in widening of the wet area and a reduction in the contact angle. When a hexadecane droplet was placed on the surface of the FAAO-incorporated material, the hydrophilic units of FAAO were pushed flat on the surface, allowing fluoroalkyl units (F) to assume erectile positions making the surface more oil-repellent. Therefore, increasing the concentration of FAAO in the coating materials led to enhancement of oil repellency of the material surface.

Biofilm formation on the FAAO-incorporated coating material was carried out using S. mutans artificial biofilms. The biofilms that were grown inside the oral simulator were stable like those in several other in vitro model systems. One of the advantages of the present in vitro oral model system was the ability to perform biofilm adherence tests on samples with different surface properties under culture conditions similar to that of the oral environment. The present results indicated that the amount of biofilm retained on the coating material gradually reduced when the concentration of FAAO was increased, suggesting that FAAO influenced biofilm formation. However, there were no significant differences among the groups. It might be noteworthy that in this study, only a limited number of specimens could be used in each experiment to restrict chemical contamination between the adjacent samples in the OBR—and this might have partially affected the results.

The process of biofilm formation on a solid surface involves several progressive stages in the oral situation. The initial stage is formation of a conditional film on the solid surface, which is mainly composed of salivary proteins and cell-free enzymes. Therefore, the specimen surfaces of the present study were inoculated with sterile saliva for 30 minutes to obtain a coating of salivary pellicle. Following this, biofilm growth was carried out on the salivary pellicle-coated surface in the OBR.

It has been reported that mutans streptococci possess low surface free energy. Hence, it was speculated that S. mutans would be repelled when the cells come within contact range of the interface due to the motile movements of hydrophobic RF units. However, when PBS nears the surface (e.g., during shaking), the hydrophobic RF units would be replaced by the hydrophilic units allowing almost frictionless running of water along the interface. As a result of this phenomenon, biofilm formation by S. mutans might be hindered. However, it was not clear whether the flip-flop phenomena really had an effect on biofilm formation in the present study.

It was recently reported that superhydrophobicity in air is characterized by a high apparent contact angle of a water droplet and a low roll-off angle. These criteria cannot be applied to underwater superhydrophobicity, since they are meaningless for a plate dipped into a liquid. In practice, the same applies to oral contact angles measured in air showing the oil repellency of the FAAO coat. Throughout the period of biofilm formation, the samples remained in an underwater condition which was essential for biofilm culturing. Since the samples remained in an underwater condition throughout the period of biofilm formation, the hydrophilic units of FAAO were probably continuously attracted to the surface.

Sucrose-induced S. mutans biofilms contain extracellular glucan polysaccharides. The production of glucans promotes accumulation of S. mutans and strong adherence to solid surfaces. In the present study, the water-insoluble glucans must have protected the formed biofilms even after vigorous shaking.

Perhaps, the current condition for FAAO incorporation into coating materials is not directly applicable for clinical applications. Nevertheless, this study was the first step to developing a new coating material with the notion of self-cleaning surface property in dentistry. The current FAAO-incorporated coating materials are not directly applicable for clinical use. Further in vitro studies should be carried out to improve material properties and durability, and elucidate biofilm formation behavior on FAAO-incorporated coating materials using multi-species bacteria.

ACKNOWLEDGEMENTS

This work was supported by the Center of Excellence Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone, Tokyo Medical and Dental University, Tokyo, Japan, and by a Grant-in-aid from the Japan Society for the Promotion of Science (JSPS; No. 16390544). We would also like to express our gratitude to Mr. Akihiko Watanabe, Department of Organic Materials, Institute of Biomaterials and Bioengineering, for his contribution in obtaining contact angle data and his assistance with the coating technique.

REFERENCES


