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RESEARCH ARTICLE

DETACHMENT OF POLYMICROBIAL BIOFILMS ON GLASS SURFACE TREATED WITH SURFACE MODIFIERS CONTAINING FLUOROCARBON CHAIN

[1]Ayako Teranaka, [2,3]Kiyoshi Tomiyama, [3,4]Kaori Miyake, [3,4]Katsura Ohashi, [5]Tota Shimizu, [1]Hirotoshi Iwai, [6]Nobushiro Hamada, [7]Norio Yoshino, [2,3]Yoshiharu Mukai, [2,3]Toshio Teranaka, [1]Satoshi Hirayama and [3,4,*]Tomotaro Nihei

[1]Department of Operative Dentistry, Nihon University School of Dentistry at Matsudo, Chiba, Japan
[2]Division of Cariology and Restorative Dentistry, Dentistry for Oral Interdisciplinary Medicine, Graduate School of Dentistry, Kanagawa Dental University, Kanagawa, Japan
[3]Institute of Oral Regenerative Medicine, Kanagawa Dental University, Japan
[4]Division of Clinical Biomaterials, Department of Oral Science, Graduate School of Dentistry, Kanagawa Dental University, Kanagawa, Japan
[5]Division of Prosthodontics and Oral Implantology, Dentistry for Oral Interdisciplinary Medicine, Graduate School of Dentistry, Kanagawa Dental University, Kanagawa, Japan
[6]Division of Microbiology, Department of Oral Science, Graduate School of Dentistry, Kanagawa Dental University, Kanagawa,Japan
[7]Departmentof Industrial Chemistry, Tokyo University of Science, Tokyo, Japan

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ABSTRACT

The purpose of this study was to evaluate the adherence and detachment of bacterial biofilms formed on glass surface modified with 10F2S-3I or FF01 using a PMbiofilm model. Modified and unmodified cover glasses were immersed in buffered McBain medium containing stimulated saliva, and theywere cultured to develop PM biofilms. There were separated six experimental groups (n = 6), designated WC: cover glass washing withCPW, WI: modified with 10F2S-3I cover glass washing with CPW, WF: modified with FF01 cover glass washing with CPW, NC: cover glass without washing, NI: modified with 10F2S-3I cover glass without washing, NF: modified with FF01 cover glass without washing.The modified and unmodified groups were further divided by either gently washing withCPW or without washing. Serial dilutions were conducted of the bacterial cultures, which were then cultured on blood agar medium to count CFU.PM biofilms were easily detached from 10F2S-3I and FF01 modified surfaces by washing with CPW. There were no significant difference of CFU among NC, NI and NF. But WI and WF were significantly lower than in WC. It was concluded that PM biofilms grown on the glass surface modified were easily detached compared with the unmodifiedglass groups.

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INTRODUCTION

Owing to the low birthrate and aging issues, importance of providing an oral care method which preferably cleans soil in the mouth is increasing to prevent the occurrence of aspiration pneumonia, endocarditisa and other diseases that can severely diminish quality of life (Abe *et al*., 2006; Paju, 2007; Mojon, 2002). However, the self-care ability of the elderly and aged people who need nursing care is low. Furthermore, the provision of proper oral hygiene by non-dental professionals, such as their family members and caregivers, is not an easy task. As a result, poor oral hygiene among such peopleare frequently seen (Petersen *et al*., 2010). In addition, plaque accumulation on dentures increases the risk of aspiration pneumonia (Yoneyama *et al*., 2002), one study reported that 70% of pneumonia in elderly people is considered to be

Corresponding author: **Tomotaro Nihei,**
[3]Institute of Oral Regenerative Medicine, Kanagawa Dental University, Japan
[4]Division of Clinical Biomaterials, Department of Oral Science, Graduate School of Dentistry, Kanagawa Dental University, Kanagawa, Japan.

aspiration pneumonia (Teramoto *et al*., 2008). Aspiration pneumonia is one of the leading causes of death in elderly, andit can be prevented by proper oral care (Scannapieco, 2014). Therefore, preventing plaque accumulation could reduce the risk of aspiration pneumonia and endocarditis. Plaque accumulation begins with attachment of bacteria on a thin layer of salivary pellicle on the tooth surface. Pellicle forms immediately, even after proper tooth cleaning, and the following plaque formation is inevitable. It is known that attachment of pellicle and plaque is affected by surface free energy (SFE) on the solid surfaces (Glantz, 1971; Van Dijk *et al*., 1987; Quiynen *et al*., 1989). Busscher *et al.* (1984) reported that when SFE on tooth surfaces is lower than 50mN/m, plaque adherence should decrease. Thus, modification of the tooth surface to lower SFE is considered an effective means to decrease plaqueformation.

We have conducted to develop a surface modifying agent to reduce bacterial adherence, plaque formation, and demineralization of tooth surface to assist in the prevention of caries and periodontal disease (Ogiwara *et al*., 1995; Kurosaka *et al*., 2000; Omoto *et al*., 2005; Nihei *et al*., 2013). Ogiwara *et al*. (1995) and Kurosaka *et al*. (2000) reported that resin composite and tooth surfaces modified by a silane coupling agent with high water and oil repellency, 1H,1H,2 H, 2H-henicosafluorododecyltriisocyanatosilane (10F2S-3I), developed by Yoshino *et al.* (1996; 1997), discouraged adherence and promoted detachment of *Streptococcus mutans*. While antibacterial studiesusing a few species of bacteria have made a dramatic advance, there are few studies on oral biofilms including many species of bacteria. In this study, polymicrobial (PM) biofilm modeldeveloped by Exterkate *et al.* (2010) was used. The purpose of this study was to evaluate the adherence and detachment of biofilms formed on glass surfacesmodified with 10F2S-3I and newly developed heptadecafluorodecylhydroxyethylmethacrylate (FF01) using PM biofilm model (Exterkate *et al*., 2010; Macbain, 2009; Exterkate *et al*., 2014; Tomiyama *et al*., 2015; Hasegawa *et al*., 2015).

MATERIALS AND METHODS

Surface modifying agent

10F2S-3I (Tokyo University of Science,Tokyo, Japan)prepared at a concentration of 3mmol/Lwith hydrofluoroether (Novec HFE 7200, 3M, Tokyo, Japan) and newly developed FF01 (Daikin Industries, Ltd., Osaka, Japan)were used as a surface modifying agent. (Table 1)

Table 1. Formula amd code of modifiers user in this study

Formula	Code
$F(CF_2)_{10}$-$(CH_2)_2$-$Si(NCO)_3$	10F2S-3I
$CH_2=CH-\overset{\overset{O}{\|}}{C}-O-CH_2-CH_2-C_8F_{17}$	FF01

Preparation of the saliva sample

Saliva was collected under ice application from a healthy adult donor, with natural dentition without active caries or periodontal disease. The inclusion criteria were as follows: not having administered antibiotics or antibacterial agents for 3 months, not having brushed their teeth for the last 24 hours, and not having had anything to eat or drink since 2 hours before saliva collection. Subjects chewed paraffin wax (Parafilm M Barrier Films, Pechiney Plastic Packaging, Chicago, IL, USA), and the stimulated saliva was collected into a plastic container which was cooled down with ice. The saliva was kept at 80°C after being diluted into a 70 vol% solution with glycerol following filtration by glass wool (NRK GRW-10, Nippon Rikagaku Kikai CO.LTO., Tokyo, Japan). The study protocol was approved by the Institutional Review Board of Kanagawa Dental University (Approval number: 206).This study was ensured compliance with the Helsinki Declaration for the consideration of the human rights of research subjects and informed consent.

Glass coverslips modifications

Fifty-fourglass coverslips (diameter 12mm, thickness 0.15mm, Menzel, Braunschweig, Germany) were soaked in 1mol/L sodium hydroxide and 1mol/L hydrochloric acid for one day each to remove contaminants. Each eighteenof the glass coverslipswere immersed in 3mmol/L10F2S-3I (group I) and FF01 (group F) for 1 hour to allow surface modification. After modification, the glass coverslips were rinsed with hydrofluoroether and dried at room temperature. The remaining 18 glass coverslips were unmodified (group C) as control. All glass coverslips were mounted on the lids of 24-well culture plates and autoclaved at 121ºC for 15 minutes.

Measurement of contact angle and SFE

Contact angle measurements employing the drop technique were carried out at 25ºC using distilled water and diiodomethane (Wako, Osaka, Japan) by automatic contact angle meter (DCA-VZ Type, Kyowa Interface Science Co, Saitama, Japan). Contact angle data on a given surface yields the surface free energy by SFE calculation software using the Owents /Wendt theory.

PM biofilms formation

Retained saliva was diluted 50-fold with buffered McBain semidefined medium (0.2wt% sucrose, 50 mmol PIPES, 2.5 g/l mucin, 2.0 g/l Bacto peptone, 2.0 g/l Trypticase peptone, 1.0 g/l yeast extract, 0.35 g/l NaCl, 0.2 g/l KCl, 0.2 g/l $CaCl_2$, 0.001 g/l hemin, and 0.0002 g/l vitamin K_1) (18-22). After medium was prepared, biofilms were produced by adding 1.5 ml of the inoculation medium with saliva to each well of standard polystyrene 24-well plates (multiwell plates, Greiner Bio One, Muunich, Germany), and the glass coverslipsfixed ontothe dedicated lids of the culture plates were immersed and anaerobically cultured for 10 hours at 37ºC (10% CO_2, 10% H_2 and 80% N_2).

Table 2. Abbreviations of combined treatments for CFU and SEM

	wash	non-wash
non-modification	WC	NC
10F2S3I	WI	NI
FF01	WF	NF
		CFU: n=6 SEM: n=3

Thereafter, the glass coverslips were anaerobically cultured for 14 hours in buffered McBain semidefined medium without saliva in the same manner. After completion of culturing, halves of group I, F and C specimens (n=9 each) were soaked

into the 24-well plates with antibiotic agents or DW for 5 minutes at 22°C.

Table 3. Contact angle and SFE for each group

	Contact angle (°) (S.D.) distilled water	dioodomethane	Surface free energy (mN/m) (S.D.)
Control (C)	14.7 (3.8) a	50.5 (1.8) a	70.5 (2.2) b
10F2S-3I (I)	114.4 (2.8) b	98.7 (3.5) b	9.5 (1.6) a
FF01 (F)	108.7 (0.9) b	90.9 (5.3) b	12.8 (1.6) a

(One-way ANOVA and Post-Hoc Tukey's multiple comparison tests) (n=6)

To wash off various antibiotics, plates were soaked in wells into which 2 ml of cysteine peptone water (CPW) was added; then, the plates were shaken up and down 10 times within 10 seconds. This process was repeated three times. The remaining halves of group I, F and C were without washing. The combinations for CFU and SEM studies were shown in Table 2.

Calculation of viable cell counts formed on glass coverslips of each group

Six each from WC, WI, WF, NC, NI and NFglass coverslipswith biofilms were transferred into tubes containing CPW at 2 ml, and subjected to ultrasonic wave for 90 seconds (Transsonic T780, Elma electric GmbH, Stuttgart, Germany), followed by vortexing for 30 seconds (Tube mixer, VTX-3500, LMS, Tokyo, Japan) to disintegrate and disperse bacterial cells. Conducting serial dilutions using CPW, 50µL of each sample was cultured in tryptic soy blood agar medium at 37ºC under anaerobic conditions (10% CO_2, 10% H_2 and 80% N_2) for 4 days, after which CFUs were counted.

SEM observation

The glass coverslips with PM biofilms of each group (n=3) were soaked in 0.01mol/Lphosphate buffered saline (PBS) for 30 seconds followed by a 30 seconds wash with 0.1mol/L cacodylate buffer. The glasses were then stored in a mixture of 0.1 mol/L cacodylate buffer and 1% glutaraldehyde for 1 hour for fixation. Each glass was then rinsed twice (30 seconds each) with 0.1 mol/L cacodylate buffer, and then, immersed in serial concentrations of ethanol solutions (50, 70, 80, 90, and 100%) for 15 minutes each at room temperature. After they were immersed in isoamyl acetate, conducted drying at the critical point. The glasses were fixed on a brass stage with a carbon tape and sputter coated with platinum (200Å in thickness). The glass surfaces were observed using a scanning electron microscope (SEM, JSM-820, JEOL, Tokyo, Japan) at an accelerating voltage of 5.0kV.

Statistical analysis

The average and standard deviation of the experimental data were calculated, and subjected to one-way ANOVA and Tukey multiple comparison assay. The level of significance was taken as p <0.05. All statistical analyses were performed by IBM SPSS Ver. 20.0 software (IBM Japan, Ltd., Tokyo, Japan).

RESULTS

Contact angle and SFE

Contact angle and SFE for each group are displayed in Table3. Contact angle of distilled water were14.7°, 114.4° and 108.7° in group C, I and F respectively. Contact angle of dioodomethane were 50.5°, 98.7° and 90.9° in group C, I and F respectively. Contact angle of distilled water for group I and F were significantly higher than group C (p<0.05). Contact angle of diiodomethane for group I and F were significantly higher than group C (p<0.05). SFE of each group were shown 70.5mN/m at group C, 9.5mN/mat group I, and 12.8mN/mat group F. Surface modified group I and F were 50mN/m or less. It was significantly lower compared to group C (p<0.05. Live bacterial count of PM biofilms on each groupare displayed in Figure 1.

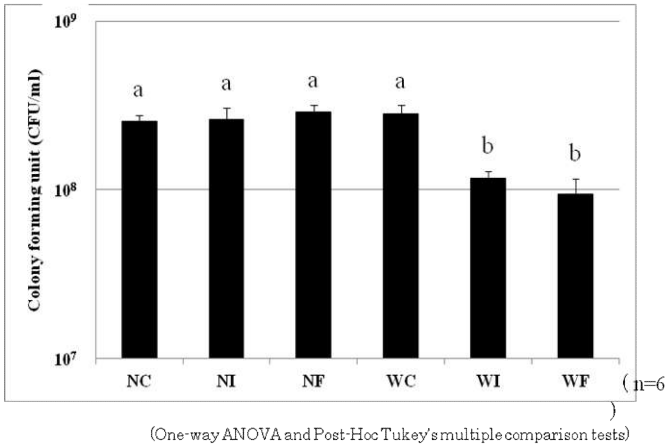

Fig.1. CFU counts of PM biofilm for each group there was no significant difference in the CFU counts between NC, NI, NF and WC (p>0.05). However, the CFU count of WI and WF was significantly lower that that of WC (p>0.05)

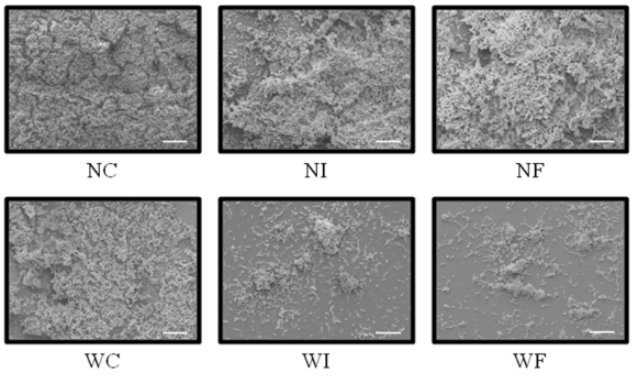

Fig.2. Scanning electorn micrographs of the PM biofilm of each group WI and WF demonstrated abacteria compared to the other groups. Bars; 10µm

The CFU counts of NI, NF and WC were similar (2.56×10^8, 2.62×10^8, 2.88×10^8 and 2.82×10^8 CFU/disk, respectively). However, the CFU counts of WI (1.17×10^8 CFU/disk) and WF (9.48×10^7 CFU/disk) were significantly lessthan those of other groups (p<0.05).

SEM observation

Images of PM biofilms attached to the glass coverslipsare presented in Figure 2. The glass surface ofWI and WF were showeddecreased bacteria than the other groups.

DISCUSSION

Dental plaque is a typical biofilm that consists of multiple species of bacteria. Biofilm formation begins with adherence

of floating microorganisms as initial colonizer in the oral fluid to the pellicle. Formation of initial colonies on the surface and subsequentaccumulation, growth microorganism leads to the development of a mushroom-shaped mature biofilm. The slimy main body of a biofilm consists of extracellular polysaccharide (EPS). Oral bacteria synthesize EPS to develop a population (Xiao *et al*., 2012). The PM biofilm used in the present study is a novel, high-throughput and active attachment model (Hung *et al*., 2012). It is an effective method of forming biofilms consisting of multiple bacterial types similar to those typically found in the oral cavity under the same conditions; this is done by storing stimulated human saliva at 80°C following sterilized glycerin preparation and by defrosting and using it for each test (Exterkate *et al*., 2010).

In addition, the method can easily be implemented, and therefore, the present biofilms consisting of multiple bacterial types are the most suitable biofilm model for the investigation of antibiotic agents, dental materials, and surface modifiers. The adherence test of PM biofilms on glass surfaces modified by 10F2S-3I and FF01 revealed no significant differences in the CFUs between NC, NI and NF, both groups were not washed with CPW, indicating 10F2S-3I and FF01 haveno antibacterial activity. After extremelygentle washing with CPW, WCwas showed no significant difference in the CFU counts with NC, NI and NF, whereas the CFU counts for WI and WF were significantly lower. It was found that after modification with 10F2S-3I and FF01, PM biofilmsadhered to the glass surface became more prone to detachment by extremely gentle washing with CPW. Although insoluble glucan is produced from sucrose at the interface, it is considered that bacteria are not able to establish a strong adherence to the modified surfacebecause of the high water and oil repellency of 10F2S-3I (Yoshino et al., 1996; Yoshino and Teranaka, 1997). These findings suggested that the surface modification effect of 10F2S-3I and FF01 are maintained even after completion of culturing. Moreover, effective detachment of the biofilm would be obtained due to the continuous modification effect after water storage for long periods of time.

Yoshino *et al*. (1996, 1997) demonstrated that surface modification with 10F2S-3I showed excellent oxidation and acid resistance against hotnitric acid. Ogiwara *et al*. (1995) and Kurosaka *et al.* (2000) reported that glass and resin composite surfaces modified with the 10F2S-3I inhibitedthe attachment and promoted detachment of *S. mutans*. Furthermore, Omoto *et al*. (2005) and Nihei *et al.* (2013) found that 10F2S-3I applied to glass, hydroxyapatite and enamel maintained high water and oil repellency in water at 37°C for 90 days, indicating that there is no notable change of surface free energy values after the completion for 24hours culturing. Also Omoto *et al.* (2005) reported that high SFE was maintained even if it was kept underwater for a long period. Consequently,it is considered that 10F2S-3I obtained high detachment of biofilms. Newly developed heptadecafluorodecylhy droxyethylmethacrylate (FF01) has characterized antifouling, antifouling edurance, water and oil repellency, excellent slidingproperty, and low effect on optical property. Chemical plaque controlling agents such as mouth rinse have been considered as a secondary preventive measure in addition to mechanical control (Baehni, 2003; Michael, 2006; Marsh, 2010), and several studies have reported that the bactericidal effect of mouthwash inhibit the increasing of biofilm thickness (Stewart, 2001; Watson *et al*., 2005; Sliepen *et al*., 2010; Yamaguchi *et al*., 2013; Wakamatsu *et al*., 2014). Even if bacteria in a biofilm are disinfected, the remaining matrix structure consisting of EPS can act as a scaffold for formation of new biofilms (Takenaka *et al*., 2012). Because 10F2S-3I and FF01were facilitated the detachment of bacteria with gentle washing, and adhered bacteria are removed by regular oral hygiene, we suggest that application of 10F2S-3I and FF01arean effective measure for preventing caries and periodontal disease. And, 10F2S-3I and FF01 may have an application as an oral care measure for used by evacuees in the event of a large-scale disaster. However, for applying FF01 in a clinical setting, it is necessary to evaluate the cytotoxicity and biocompatibility of FF01. In molecular structure of FF01, it is considered possible that to add to polymerization initiators, forming a bond with intermolecular andforming ultrathin film polymer or add to crosslinking monomer. It was considered possible development in water repellent monomer. In the future, it is necessary to examine the physical properties and durability of these modifiers.

Conclusion

It was concluded that after modification with 10F2S-3I and FF01, PM biofilms adhered to the glass surface became more prone to detachment by extremely gentle washing with CPW. From these results, it was suggested that it was the modifier which kept intraoral environment well.

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