An analysis of the biofilms adhered to framework alloys using *in vitro* denture plaque models

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The purpose of this study was to perform a quantitative and qualitative evaluation of the biofilms formed on framework alloys *in vitro*. The biofilms formed by unfiltered fresh human saliva or *Streptococcus mutans* and/or *Candida albicans* on commercially pure titanium and gold-copper-platinum demonstrated higher amounts than other alloy and resin samples. In contrast, the silver-palladium-copper-gold showed a significantly decreased level of biofilm formation. Although the adhesion level of *Streptococcus mutans* on cobalt-chromium was high, that of *Candida albicans* was less extensive. A T-RFLP analysis and qualitative PCR of the microbes in the biofilms were performed. In a cluster analysis of all T-RFLP profiles, the cobalt-chromium pattern was integrated into one cluster. On qualitative PCR, the existence of microorganisms related to caries, periodontitis and aspiration pneumonia was observed. Our results showed that the biofilm formation on each framework alloy was different in terms of both the quantity and quality.

Keywords: Framework alloy, Denture plaque, T-RFLP, Microbiota

INTRODUCTION

The wearing of removable dentures can lead to the colonization of oral pathogenic bacteria and/or poor oral hygiene¹⁻³. Dentures become a reservoir for dental plaque, which can cause local and systemic diseases⁴⁻⁶. The health population movement statistics in Japan (2011) showed that the incidence of pneumonia has been increasing as the cause of death in Japan, and now exceeds cerebrovascular disease⁷.

The major pathogenic bacteria associated with aspiration pneumonia are oral bacteria, particularly *Peptostreptococcus*, *Prevotella* and *Fusobacterium*⁸. Aspiration pneumonia is also caused by denture plaque^{5,6,9} that contains the same anaerobic bacteria, such as *Prevotella* and *Fusobacterium*⁹. Oral care, with a focus on oral hygiene to reduce the dental plaque, can help decrease the incidence of pneumonia^{10,11}. Among the microorganisms present in denture plaques, *Candida albicans* (*C. albicans*) and *Streptococcus mutans* (*S. mutans*) are well known as pathogens causing oral infections^{2,12-16}. The level of *C. albicans* increases in the oral cavity following the insertion of dentures, and can cause denture stomatitis, aspiration pneumonia and intestinal infections^{2,12-14}. *S. mutans* is a caries-causing oral *Streptococci*^{15,16}, and is associated with inducing dental caries on the remaining teeth in the oral cavity of denture wearers.

Denture plaque easily adheres to the resin denture base¹⁷, because the surface of the acrylic resin is

porous. Another problem associated with resin dentures is their weak mechanical strength when compared to metal base dentures. Metal base dentures have excellent mechanical strength¹⁸. Cobalt-chromium alloy (Co-Cr) is mainly used for the metal framework of removable dentures. Recently, commercially pure titanium (CP Ti) and titanium alloys (Ti 6-7) have been used as the metal framework of removable dentures¹⁹. Titanium has excellent biocompatibility, low allergic potential^{20,21}, lower thermal conductivity and greater laser beam absorption than other metal alloys²², making it an ideal material for dentures and other medical appliances.

There have been some reports of the quantitative evaluation of adhesion plaques and the qualitative bacterial colonization on the metal materials used for prostheses^{23,24}. For example, Yamane *et al.* investigated the bacterial adhesion affinities on various implant abutment materials²³. They concluded that Au-Pt alloy accumulated less plaque than other materials. On the other hand, there have been no reports that have performed both quantitative and qualitative studies of the adhered biofilms on various framework alloys.

The purposes of this study were to analyze the biofilms adhered to the framework alloys both in quality and quantity of microbiota, and to compare each alloy and conventional resin.

MATERIAL AND METHODS

Fabrication of alloy samples

The rectangular alloy samples (20.0×12.0×1.4 mm) were cast using the materials shown in Table 1. The surface of each alloy sample was polished to be a mirror-smooth surface in accordance to the polishing procedure used by the Tsurumi University Hospital Laboratory Center²⁵⁾, and was sterilized by an autoclave. As a control, the same size of resin sample was prepared with polymethyl methacrylate (PMMA) self-curing denture base resin (resin, Palapress vario, Heraeus-kulzer, Hanau, Germany), sterilized with 80% ethanol, which was immersed in distilled water for 24 h.

Measurement of the surface properties

The average surface roughness of all samples was measured with a non-contact coordinate measuring machine (NH-4H, Mitaka Kohki Co., Ltd., Mitaka, Japan). The surface contact angle and an index of hydrophilicity, was also measured using a contact angle-measuring apparatus (CA-P, Kyowa Interface Science Co., Ltd., Niiza, Japan).

Observation of the surface of the samples

The surface of the samples after being immersed in the filtered saliva overnight was observed using a scanning electron microscope (SEM; JSM-5600LV, JEOL Ltd., Akishima, Japan) and an electron probe microanalyzer (EPMA: JXA-8900RL, JEOL Ltd). Only resin samples

were sputter-coated with gold to a thickness of approximately 250 Å in a vacuum evaporator.

Preparation and cultivation of biofilm-adhered samples

In order to make an *in vitro* denture plaque model using the framework alloys and resin, biofilms were formed on each sample using human saliva. Stimulated human saliva was collected by the chewing gum method from volunteer subjects, and was divided into non-filtered and filtered samples. The study protocol was approved by the ethics committee of Tsurumi University (permission no. 1040), and all volunteers provided informed consent for their participation. The subjects who had untreated dental caries, removable dentures or periodontitis were excluded. The selected subjects were five adult healthy males between 29 and 31 years old who had not used any antibiotics for the past three months.

Fifteen samples of each alloy were immersed immediately in the non-filtered human saliva and were continuously incubated under aerobic conditions at 37°C overnight to allow early biofilms to form on the samples (Fig. 1). On the second day, the samples were removed from the saliva and immersed in brain-heart infusion broth (BHI; Difco, Becton, Dickinson and Company, Sparks, MD, USA) for the maturation of the biofilms. The BHI broth was replaced every day until the seventh day.

*Preparation of biofilms with *S. mutans* or *C. albicans**

The precultures of *S. mutans* and *C. albicans* were

Table 1 The materials used in this study, their composition and their surface properties

	Abbreviation	Trade name / distribution source	Element, wt%	Surface roughness (μm)		Contact angle (°)	
				Uncoated	Saliva-coated	Uncoated	Saliva-coated
Commercially pure titanium	CP Ti	T-alloy H / GC Corp., Tokyo, Japan	Ti, 99.2	0.045±0.009	0.044±0.008	93.5±2.1	36.0±4.7
Titanium alloy	Ti 6-7	T-alloy Tough / GC Corp., Tokyo, Japan	Ti, 86.5; Nb, 7.0; Al, 6.0	0.040±0.008	0.042±0.005	93.5±1.8	35.2±3.3
Cobalt-chromium alloy	Co-Cr	Wisil M / Elephant Dental B.V., Hoorn, Netherland	Co, 63.1; Cr, 28.0; Mo, 6.0	0.044±0.011	0.048±0.007	93.6±2.5	34.4±3.6
Silver-palladium-copper-gold alloy	Ag-Pd-Au	Kinparabest12 / Ishifuku, Tokyo, Japan	Ag, 49.5; Pd, 20.0; Cu, 19.0; Au, 12.0,	0.035±0.005	0.038±0.008	93.2±3.4	32.4±2.6
Gold-copper-platinum alloy	PGA	PGA-3 / Ishifuku, Tokyo, Japan	Au, 70.0; Cu, 19.0; Ag, 4.7; Pt, 6.0,	0.035±0.008	0.038±0.007	95.9±2.6	33.2±1.8
Denture base resin	resin	Palapress vario / Heraeus-kulzer, Hanau, Germany	Polymethyl methacrylate	0.082±0.008	0.082±0.013	70.4±3.0	34.0±4.0

Fig. 1 Preparation and cultivation of *in vitro* denture plaque models, and measurement of the biofilm levels.

carried out as follows: *S. mutans* ATCC25175 was precultured in brain-heart infusion broth (BHI; Difco, Becton, Dickinson and Company, Sparks, MD, USA) at 37°C in an anaerobic chamber. An aliquot of the *S. mutans* culture was inoculated into 10 mL of BHI. The number of *S. mutans* cells was approximately 1×10^7 to 1×10^8 CFU/mL. *C. albicans* ATCC18804 was precultured in tryptic soy broth supplemented with 5% dextrose (TSBD; Difco, Becton, Dickinson and Company) medium at 37°C with aerobic shaking. An aliquot of 300 μ L of the precultured *C. albicans* was inoculated into 3 mL of TSBD. The number of *C. albicans* cells was approximately 1×10^6 to 1×10^7 CFU/mL.

For the quantitative analysis of biofilms formed by *S. mutans* or *C. albicans*, ten samples of each alloy was immersed in the filtered pooled saliva overnight at 4°C (Fig. 1). The samples were divided into two groups, five samples were immersed in *S. mutans* culture solution, and the other five samples were immersed in *C. albicans* culture solution for one day.

Measurement of the wet-weight, living bacteria and extracellular polysaccharide (EPS) in biofilms

As shown in Fig. 1, on the first, fourth and seventh days of cultivation, five samples of each alloy or resin were taken from the media, and the wet-weight was measured after removing the excess media. Each sample was then incubated in RPMI 1640 medium supplemented with 10% FBS and a REDOX indicator (Alamar blue; Trek Diagnostic Systems, Cleveland, OH, USA) at 37°C to estimate the amount of living bacteria present in the biofilm. The amount of living bacteria in the biofilm was

determined based on the fluorescence levels (EX: 530 nm, EM: 590 nm) using a fluorescent microplate reader, the Cyto-Fluor 2 (Per Septive Biosystems, Farmingham, MA, USA). The cultivation was stopped when the fluorescence of the sample reached a value of 30,000.

Following the estimation of the amount of living bacteria, the amount of EPS was determined by dental plaque staining (Dent liquid plaque tester, LION Co., Ltd., Tokyo, Japan). The stain-incorporated EPS was extracted with 99.9% ethanol, and the optical density of the extracts was measured using an absorbance meter (UV-1200, SHIMADZU Co., Ltd., Kyoto, Japan) at OD562 nm to examine the quantity of EPS²⁶⁾.

The same quantitative analyses were also performed for the biofilms formed by *S. mutans* or *C. albicans* on five samples of each alloy or resin after overnight cultivation.

The terminal restriction fragment length polymorphism (T-RFLP) analysis

To perform a T-RFLP analysis of the biofilms, each alloy or resin sample was incubated with fresh unfiltered individual saliva ($n=5$) overnight. Each sample was washed in distilled water three times to remove non-adherent microbial cells. The genomic DNA was extracted from each of the samples, and from individual saliva samples, with the QIAamp DNA Mini Kit (QIAGEN, Tokyo, Japan), and was stored at -20°C .

The internal regions of 16S rRNA genes were amplified from genomic DNA using the universal forward primer, FAM (5'-AGA GTT TGA TYM TGG CTC AG-3'), labeled at the 5' end with 6-carboxyfluorescein

(6- FAM) and the universal reverse primer, HEX (5'-GGA CTA CCR GGG TAT CRA A -3'), labeled at the 5' end with hexachlorofluorescein. The PCR was performed with 5 μ L of sample added to 45 μ L of reaction mixture containing 5 μ L 10 $\times$ Ex taq buffer (TaKaRa EX Taq, TaKaRa Bio Company, Ohtsu, Japan), 0.25 μ L 5U/ μ L Taq polymerase (TaKaRa ExTaq, TaKaRa Bio Company) and 20 pmol/ μ L of each of primer. PCR amplification was performed in a thermal cycler (PCR Thermal Cycler MP, TaKaRa Bio Company). The PCR temperature profile was an initial denaturation step at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 30 s and extension at 72°C for 1.5 min, and final step of 72°C for 10 min.

The PCR amplification, purification and digestion with *Hae* III, *Hha* I and *Msp* I were conducted as described previously²⁷. The restriction digest products were denatured, electrophoresed and analyzed using the GeneMapper software program, version 4.0 (Applied Biosystems, Foster City, CA, USA). Terminal restriction fragments with peak areas < 0.5% of the total area were eliminated from the analysis.

Qualitative PCR for oral microorganisms

Qualitative PCR was carried out according to the previous studies^{28–40} using DNA templates extracted from the biofilms on five alloys, resin and the individual saliva samples ($N=5$). The primers used were for the genera of *Actinomyces*²⁸, *Burkholderia*²⁹, *Fusobacterium*³⁰, *Haemophilus*³¹, *Mycoplasma*³², *Neisseria*³³, *Peptostreptococcus*³⁴, *Pseudomonas*³⁵, *Streptococcus*³⁶, *Staphylococcus*³⁷, and *Candida*³⁸ and for the species of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Treponema denticola*, *Tannerella forsythia*³⁹ and *S. mutans*⁴⁰.

Statistical analysis

The surface properties (surface roughness and contact angle) were analyzed using a one-way analysis of variance (ANOVA) and Tukey's test, with a significance level set at $\alpha=0.05$. The statistical analyses of the quantitative analyses of the biofilms were performed for multiple groups using the Kruskal-Wallis test, and between two groups using a Mann-Whitney U-test, with the significance level set at $\alpha=0.05$. A cluster analysis was performed using Ward's algorithm (SPSS 14.0, IBM Japan, Ltd., Tokyo, Japan). The dissimilarity analysis of the T-RFLP data was carried out with a Bray-Curtis analysis (SYSTAT13, Systat Software, Inc., Chicago, IL, USA).

RESULTS

Measurement of the surface properties

The surface roughness and contact angle of each sample coated with filtered saliva was within the range of surface roughness 0.04–0.05 μ m and contact angle 32–36°, respectively. There were no significant differences in the surface roughness and contact angle among

the five alloys, except for the control resin (surface roughness 0.08 μ m, contact angle 34°). The contact angle of the samples with saliva coating was significantly decreased (Table 1).

Observation of the surface of each sample

The samples coated with the filtered saliva were observed by SEM and EPMA. The surface of each sample in SEM was different. Certain substances derived from saliva were observed on all of samples except for Ag-Pd-Au (Fig. 2a). The intensity on the surface of the gold-copper-platinum alloy (PGA) in EPMA images of calcium was higher in comparison to the other samples (Fig. 2b).

Quantitative analysis of the biofilms

The levels of biofilm adherence to each sample was determined based on three parameters; the wet-weight, the activity of living bacteria and the amount of EPS in the biofilm. The results of these analyses are shown in Fig. 3. The amounts of adherent biofilm in all of three assays increased during the incubation in a time-dependent manner. The determination of living bacterial activity was measured when the redox indicated reached its maximum value (approximately 30,000–40,000), which was after 15 h of incubation for overnight culture, 5 h of incubation after a 4-day culture and 3 h of incubation after a 7-day culture. PGA and CP Ti showed higher adherence levels than did the other alloys and resin. There were significant differences in the adherence levels between PGA or CP Ti and resin after both the overnight and 4-day incubations ($p<0.05$).

In contrast, Ag-Pd-Au showed a significantly lower value compared with the other alloys ($p<0.05$). On day 7, there were no significant differences in the amount of adherent biofilm among the alloys and resin, except for the Ag-Pd-Au alloy, which was significantly lower than the other samples ($p>0.05$).

The SEM images showed the biofilm that adhered to the CP Ti to have formed several layers by day 7 (Fig. 4).

Analysis of the microbial components by T-RFLP

The total biofilm was recovered from each sample after overnight cultivation with individual saliva samples, and DNA was extracted to perform a T-RFLP analysis. Figure 5 shows the peak area of the T-RF with the *Hae*III restriction enzyme, which was more than 1% of the total area. Peak area 33 was the major peak for all of the alloys. In this peak area, PGA showed the highest ratio, which was approximately 65%, and Co-Cr had the lowest, at 25%. Co-Cr showed a larger ratio than the other alloys for peak areas 60 and 68. Peak area 84 was the highest on Ag-Pd-Au among all of the alloys.

Figure 6 shows the profiles of T-RFLP as indicated by the cluster analysis. All samples were categorized into two main clusters (clusters I and II). Although the T-RFs of all five Co-Cr samples were classified into cluster I, four of the five PGA samples were classified into cluster II. Three samples of CP Ti, Ti 6-7 and resin

Fig. 2 a) The surface of each sample coated with the filtrated saliva observed by SEM
b) EPMA image of calcium (color bar: X-ray intensity of calcium).

were classified into cluster I, and the other two samples belonged to cluster II. The profiles of CP Ti, Ti 6-7, resin and the saliva samples (subject Nos. 1, 2, 4, 5) were closely grouped, and they were consequently classified

in the same cluster, except for saliva sample No 3.

When a Bray-Curtis analysis was performed to compare the similarity of the T-RFLP profiles, CP Ti/Ti 6-7, CP Ti/resin, and Ti 6-7/resin showed higher

Fig. 3 Determination of the amount of biofilm adhered to each alloy. The wet-weight of the adherent biofilm, the activity of living bacteria and the amount of dye inclusion were measured to assess the levels of biofilm adherence. The asterisk indicates a significant difference (Mann-Whitney U-test, $p<0.05$). A: The wet-weight of the adherent biofilm; the vertical axis shows the increase in the weight, B: The activity of living bacteria; the vertical axis shows the fluorescence level of the metabolized redox indicator ($\times 1000$), C: The amount of EPS, indicating the levels of biofilm adherence; the vertical axis shows the absorbance at 562 nm, after overnight culture in saliva (white bar), four days of culture in BHI after the overnight culture in saliva (gray bar) and seven days of culture in BHI after the overnight culture in saliva (black bar).

Fig. 4 The SEM images of biofilms on the CP Ti and Ag-Pd-Au at day 7.

Fig. 5 The T-RFLP peak distribution of the alloy, resin and saliva samples. The bars demonstrate the average levels of the peak areas of the T-RFs with *Hae* III. The cut-off value was set at 1%.

Fig. 7 The Bray-Curtis dissimilarity of the T-RFLP profiles. The differences between various sets of two types of samples are shown.

Fig. 6 The results of the cluster analysis of the T-RFLP pattern for all samples. The T-RFLP patterns of *Hae* III, *Hha* I and *Msp* I were analyzed by the Ward method. (): subject numbers.

similarity, at levels of around 0.14, as shown in Fig. 7 (indicating that they were very similar). These results were consistent with the distance between the clades in the cluster analysis. In contrast, the similarity of Co-Cr and PGA in the different clusters showed a lower level (0.52, not very similar).

Qualitative PCR

The T-RFLP analysis was performed with three enzyme; *Hae* III, *Hha* I and *Msp* I in this study. The T-RFs which showed a more than 10% peak level for all of three enzymes were selected to predict the genus of the microorganisms based on a database⁽⁴¹⁾.

To confirm these findings, a qualitative PCR analysis was carried out using genus- and species-specific primers. The genera of *Actinomyces*, *Burkholderia*, *Fusobacterium*, *Haemophilus*, *Mycoplasma*, *Neisseria*, *Peptostreptococcus*, *Pseudomonas*, *Staphylococcus* and *Streptococcus* were detected in the template DNA of the saliva samples (Table 2). On the contrary, genera of *Actinomyces*, *Fusobacterium*, *Haemophilus*, *Mycoplasma* and *Peptostreptococcus* were found in the template DNA of the biofilms on five alloys and resin. *Burkholderia*, *Pseudomonas* and *Staphylococcus* were detected in the saliva samples, but not in the five alloy and resin samples. This suggested that these three genera did not adhere to the five alloys or resin after overnight culture.

The template DNA samples in the biofilms on the five alloys and resin showed evidence of periodontal disease-causing microorganisms, including *T. denticola*, *T. forsythia*, *P. gingivalis*, *P. intermedia* and also the cariogenic bacteria, *S. mutans*. The binding properties or affinities of *Mycoplasma*, *P. gingivalis* and *T. denticola* for the five alloys and resin were not equal (Table 2).

Table 2 The qualitative PCR detection of candidate genus and species in the saliva and plaques adhered to five alloys and resin

PCR detection	Saliva	CP Ti	Ti 6-7	Co-Cr	Ag-Pd-Au	PGA	resin
Genus-specific primers							
<i>Actinomycetale</i>	5/5	5/5	5/5	5/5	5/5	5/5	5/5
<i>Burkholderia</i>	5/5	0/5	0/5	0/5	0/5	0/5	0/5
<i>Fusobacterium</i>	5/5	5/5	5/5	5/5	5/5	4/5	5/5
<i>Haemophilus</i>	5/5	5/5	5/5	5/5	5/5	5/5	5/5
<i>Mycoplasma</i>	5/5	4/5	4/5	4/5	3/5	3/5	4/5
<i>Neisseria</i>	5/5	5/5	5/5	5/5	5/5	5/5	5/5
<i>Peptostreptococcus</i>	5/5	5/5	5/5	5/5	5/5	5/5	5/5
<i>Pseudomonas</i>	5/5	0/5	0/5	0/5	0/5	0/5	0/5
<i>Staphylococcus</i>	5/5	0/5	0/5	0/5	0/5	0/5	0/5
<i>Candida</i>	0/5	ND	ND	ND	ND	ND	ND
Species-specific primers							
<i>A. actinomycetemcomitans</i>	ND	0/5	0/5	0/5	0/5	0/5	0/5
<i>P. gingivalis</i>	ND	2/5	3/5	2/5	1/5	1/5	2/5
<i>P. intermedia</i>	ND	2/5	2/5	2/5	2/5	2/5	2/5
<i>T. denticola</i>	ND	0/5	3/5	3/5	1/5	5/5	0/5
<i>T. forsythia</i>	ND	5/5	5/5	5/5	5/5	5/5	5/5
<i>S. mutans</i>	ND	5/5	5/5	5/5	5/5	5/5	5/5

(number of positives/subject, ND: not determined)

Quantitative analysis of the initial adhesion of *S. mutans* and *C. albicans* on framework alloys

Prior to the quantitative analysis, the presences of *S. mutans* and *C. albicans* in the saliva samples was examined by a culture method and qualitative PCR. *Candida* was not detected, but *S. mutans* was found (Table 2). The adhesion levels of *S. mutans* and *C. albicans* were evaluated on the saliva-coated framework alloy samples immersed in the suspensions of *S. mutans* or *C. albicans*, based on the wet-weight of the biofilms, the activity of living microorganisms and the amount of EPS (Fig. 8). The adhesion levels of biofilms of both microorganisms on Ag-Pd-Au were lower than those on the other samples. CP Ti and PGA demonstrated adhesion levels similar to or higher than those of the resin. The adherence of *S. mutans* to Co-Cr was comparable to that on CP Ti and PGA, but that of *C. albicans* to Co-Cr was significantly lower than that to CP Ti and PGA.

DISCUSSION

Previous studies have suggested that dentures can become a reservoir or hotbed of plaque, and affect the systemic health of denture-wearers⁴⁻⁶. However, in those reports, the material investigated was the resin denture, but not the framework alloys. To the best of our knowledge, the present study is the first to examine the plaque formation on both the denture resin and various framework alloys.

Surface properties of alloy and resin samples

A previous study of the topography on biofilm

development showed that the major factors affecting bacterial adherence were the surface roughness and contact angle⁴². There have been various reports about the impact of the surface roughness on the plaque adherence⁴²⁻⁴⁸. It was described that the threshold surface roughness should be 0.2 μm ⁴⁶⁻⁴⁸, and the results of the present study showed that the surface roughness of all samples was below 0.05 μm . There was no difference in the surface roughness between before and after saliva coating. And the level of surface roughness was almost that same among the alloy samples (Table 1). Therefore, we concluded that the surface roughness did not affect the biofilm adherence in this study. On the other hand, the contact angle in all samples without saliva coating was close to 90°. The contact angle in all samples with saliva coating was close to 35° (Table 1), thus indicating that the surfaces were coated with salivary components. There was no significant difference in the contact angle among the all samples. Therefore, we concluded that the contact angle did not affect the biofilm adherence in this study.

Quantitative analysis of biofilms on the samples

To evaluate the amount of biofilm present, the wet-weights of the biofilms on the samples were measured. The wet-weight reflects the biofilm volume, but might be affected by the measurement environment. Therefore, we also determined the activity of living bacteria in the biofilm and the amount of EPS in the biofilm²⁶, to more accurately assess the amount of biofilm.

Figure 3 shows the results of these three different quantitative analyses. The wet-weight, the activity of living bacteria and the amount of EPS were

Fig. 8 The determination of the amounts of *S. mutans* or *C. albicans* adhered to each alloy. After immersion in the filtered pooled saliva overnight, the alloy samples were immersed in the *S. mutans* or *C. albicans* culture suspension and cultivated for one day. The wet-weight of the adherent biofilm, the activity of living bacteria and the amount of dye inclusion were assessed to indicate the levels of *S. mutans* and *C. albicans* adhered to the samples. The asterisk indicates a significant difference (Mann-Whitney U-test, $p < 0.05$). A: The wet-weight of the adherent biofilm; the vertical axis shows the increase in the weight, B: The activity of living bacteria; the vertical axis shows the fluorescence level of the metabolized redox indicator ($\times 1000$), C: The amount of EPS, indicating the levels of biofilm adherence; the vertical axis shows the absorbance at 562 nm by *S. mutans* and *C. albicans*

approximately in the order of $\text{PGA} = \text{CP Ti} > \text{Ti 6-7} > \text{Co-Cr} > \text{Ag-Pd-Au}$ from day 1 until day 4. However, the differences disappeared on day 7, except for the finding that Ag-Pd-Au still had the lowest values.

We expected that the resin used as a control would have more adherence by biofilms than alloy samples, because resin is thought to be easily absorbed by denture plaque¹⁷⁾. However, to our surprise, the amount of adherent biofilm on the resin sample was significantly lower than that of all of the alloys except Ag-Pd-Au on both overnight and day 4 ($p < 0.05$). Kawai *et al.* previously reported the resin monomer has antimicrobial effects against oral bacteria⁴⁹⁾. There was another report indicating that the monomer was mostly eluted within 24 h after polymerization. However, the small amount of remaining monomer eluted gradually even with immersion for 24 h, and some monomer persisted for several years⁵⁰⁾. The results for the resin were therefore considered to reflect the present of the remaining monomer, which decreased the biofilm adherence on the resin surface. The significant difference

between the resin and the other alloys was lost on day 7 (Fig. 3), which may have reflected the loss of a large amount of the remaining monomer.

The quantitative analyses of the biofilms on the framework alloys indicated that there were differences in the amount of adhesion on each alloy until day 4, however, those differences were lost by day 7.

Observation of the surface of the samples

Although the contact angle of all samples was the same, some differences in the surfaces of each sample were observed on the SEM images. Especially, no salivary substances were confirmed on the surface of Ag-Pd-Au (Fig. 2a). The components of the salivary substance were thought to be proteins. Because the surface of materials exposed to the oral environment is directly converted by the spontaneous adsorption of protein-dominated films^{51,52)}. The amount and composition of such protein might have affected the amount of the biofilm. In addition, calcium ion participated in the attachment among alloy surface, saliva protein and bacteria^{53,54)}.

The amount of calcium on the surface of PGA by EPMA images was larger than that seen in other samples (Fig. 2b). Further investigations to clarify the differences affecting the amount of biofilm on the PGA are thus called for.

Due to the observed differences in the saliva protein attached to the surfaces, and also in the degree of biofilm formation, it might be necessary to analyze the composition of the saliva protein attached to each sample.

Qualitative analyses of biofilms on the samples

A qualitative analysis was performed to examine the differences in the microbial components present in the biofilms. Recently, new molecular biology techniques have made it possible to perform a metagenomic analysis of the bacterial flora, including the non-culturable bacteria. For these metagenomic analyses, denaturing gradient gel electrophoresis (DGGE)³⁾, terminal restriction fragment length polymorphisms (T-RFLP)⁵⁵⁾, next generation sequencing⁵⁶⁾ and the clone library technique⁵⁷⁾ are usually performed. Sakamoto *et al.* previously analyzed the oral bacterial flora, with non-culturable species, from patients with periodontal disease using the T-RFLP method, and compared the findings before and after periodontal treatments⁵⁵⁾. They showed that the sensitivity and specificity of the T-RFLP method were almost the same as those of real-time PCR of the 16SrDNA clone library, but the T-RFLP method was more reliable, because the apparatus can be automated by using capillary electrophoresis, and the reproducibility of the results is higher than that of the DGGE method⁵⁸⁾. Another advantage of T-RFLP is that it makes it possible to comprehensively predict the component species in the bacterial flora. Therefore, we used the T-RFLP method to analyze the bacterial components of the biofilms using an *in vitro* denture plaque model.

The peak area pattern of T-RFLP revealed that the ratio of bacterial species attached to the Co-Cr was different compared to that of the other alloys (Fig. 5). We performed a cluster analysis using the results of the T-RFLP fragments to determine the similarity of the patterns. The pattern of Co-Cr was unique in spite of using saliva samples from different individuals, and accumulated in cluster I (Fig. 6). In contrast, CP Ti, Ti 6-7 and resin showed different results for the different saliva samples. The results of the cluster analysis showed that the clade distance of CP Ti, Ti 6-7 and resin indicated that they were neighbors. Those results were similar to those of an investigation of the bacterial community on an implant abutments model reported by Yamane *et al.*²³⁾

The cluster analysis allowed the relationship of the whole variations of microbes to be visualized by comparing the similarity of all samples, and these findings could be summarized in a dendrogram. However, there are pros and cons to studies where the basis of non-similarity where the euclidean distance is based on a cluster analysis⁵⁹⁾. Therefore, we also carried

out a Bray-Curtis analysis, because it reflects the nature of the difference between subjects⁶⁰⁾. The Bray-Curtis analysis of the T-RFLP results showed that there was high similarity of T-RFLP profiles of CP Ti, Ti 6-7 and resin. These results suggest that those materials have similar levels of bacterial attachment (Fig. 7). However, there was limited similarity among CP Ti, Ti 6-7 and resin and the saliva samples themselves (non-similarity shown by the Bray-Curtis index=0.5). This result indicated that only some limited bacteria in the saliva samples might adhere to the alloys and resin.

When the signal data for three degradation enzymes was matched, the accuracy of the estimation might have been increased, and the database⁴¹⁾ of bacterial species might have better correspond to the T-RFLP pattern. Therefore, a principal component analysis of the T-RFLP profiles was performed for the three enzymes (date not shown). The results of the principal component analysis, which was performed *in silico* using statistical data and an estimate based on the ratio, showed the possibility that area 33 might contain *Neisseria* and/or *Haemophilus*. Area 33 was the most abundant peak in all samples (Fig. 5). This suggests that the predominant bacteria in all samples might be *Neisseria* and/or *Haemophilus*. Teles *et al.* previously reported that *Neisseria* was the dominant species in the dental plaque on denture teeth, as well as on natural teeth⁶¹⁾. Zhu *et al.* also reported the same finding in saliva, dental plaque and residual ridge³⁾. Ritz described the process of plaque maturation from the initial adhesion⁶²⁾. During this process, *Neisseria* comprised only 10% of the early plaque. The discrepancy between the report by Ritz *et al.* and the studies by Teles, Xhu and our present findings might be explained by the fact that they used only the culture technique. It seems worthwhile to re-examine the normal oral bacterial flora using a metagenomic analysis, because it allows for easier identification of the flora, including bacteria that are difficult or impossible to culture.

The qualitative PCR analysis using the genus- or species-specific primers was carried out to confirm whether the species narrowed down from the database⁴¹⁾ truly corresponded to the T-RF of the five alloys and resin. The results of the PCR analysis for the five alloys and the resin sample showed that several microorganisms associated with aspiration pneumonia, including genera of *Actinomyces*, *Fusobacterium*, *Haemophilus*, *Mycoplasma* and *Peptostreptococcus* (Table 2), existed in the biofilms formed on the five alloys and resin. The results of the species-specific PCR showed that *P. gingivalis*, *P. intermedia*, *T. denticola* and *T. forsythia*, all periodontal pathogens, were also identified in the five alloys and resin samples. These results revealed that various clinically-relevant pathogens attached to the five alloys and resin after just an overnight immersion in saliva. Yasui *et al.* revealed that *A. actinomycetemcomitans*, *P. intermedia*, *P. gingivalis*, *T. denticola*, *T. forsythia* and *Fusobacterium nucleatum* were found in the resin denture plaque by PCR⁹⁾. Moreover, Sumi *et al.* reported the colonization

of respiratory pathogens in denture plaques^{5,6}). These reports confirm the importance of denture cleaning to prevent the adherence of these pathogens. Interestingly, the binding properties or affinities of *Mycoplasma*, *P. gingivalis* and *T. denticola* to the five alloys was shown to be diverse. This result suggested that bacteria adhered selectively to each alloy.

The adhesion properties of S. mutans and C. albicans to the framework alloys

There have been many studies on the adhesion of *S. mutans* and *C. albicans* to resin^{1,2,12,13,63}), but few reports about their adhesion to the framework alloys. *C. albicans* increases after wearing dentures, and becomes the major pathogen responsible for denture stomatitis. There was a previous report that showed that the level of *S. mutans*, a highly cariogenic bacterium^{15,16}), also increased after wearing dentures³). Therefore, we conducted a quantitative analysis of the initial adherence of *C. albicans* or *S. mutans* using our *in vitro* model.

The adhesion level of *C. albicans* showed the same tendency as the adherence level of biofilms using unfiltered saliva alone (PGA=CP Ti>Ti 6-7> Co-Cr>Ag-Pd-Au). On the contrary, the adhesion level of *S. mutans* showed a different tendency, where the adherence to Co-Cr was almost the same as that to PGA and CP Ti (Fig. 8). This result suggested that *S. mutans* preferentially adhered to Co-Cr, PGA and CP Ti. Zhu *et al.* reported that *S. mutans* increased in the oral cavity after wearing dentures made of Co-Cr³), which was similar to our present results. The initial adhesion of *C. albicans* or *S. mutans* to Ag-Pd-Au was lower than that to PGA and CP Ti, as well as the results of the adhesion experiments with saliva. The reason for this low adhesion rate on Ag-Pd-Au was likely due to the fact that the silver ions⁶⁴) and/or the presence of less salivary protein prevented the adhesion and growth of bacteria (Fig. 2a). However, further studies are called for to clarify this phenomenon.

Factors affecting the adhesion of biofilms to the framework alloys

The alloys used in this study were divided into two groups, the precious metals included PGA and Ag-Pd-Au, and the base metals included CP Ti, Ti 6-7 and Co-Cr, which formed passive films on the surface. The PGA used in this study contained 70% Au that was specifically bond by the disulfide group⁶⁵). The observed phenomenon strongly suggested that the salivary proteins or peptides comprising disulfide ions can become interposed on the surface of the PGA. In contrast, a specific passive film of titanium, which is well known to cause osseointegration, has a negative electric charge at near-neutral pH, and can bind with positive ions such as calcium. The affinity for tissue calcium ions is thought to be related to the attachment of protein onto the surface of titanium⁵⁴), although the present EPMA analysis could not confirm the adsorption of calcium ion.

The amount of biofilm form on Ti 6-7 was less than that on CP Ti. The reason for the difference between

Ti 6-7 and CP Ti might be due to the differences in the passive film. Kelly *et al.* showed that a passive film on titanium 6Al-4V consisted of titanium and aluminum⁶⁶). The passive film of Ti 6-7 may contain aluminum, because Ti 6-7 contains both aluminum and niobium. This aluminum might affect the biofilm formation in a manner similar to that in the passive film of titanium 6Al-4V. Further investigation is necessary to clarify the component(s) of the passive film formed on Ti 6-7.

On the other hand, the passive film formed by Co-Cr has a small deflection of the charge due to close to neutral at pH 7.0⁶⁷). The relative permittivity can affect the changes in the conformation of proteins⁶⁸). The relative permittivity level of the passive film on the Co-Cr is considerably lower compared to that of titanium. Therefore, the level of adherent biofilm on the Co-Cr was lower than that on CP Ti or Ti 6-7 (Fig. 3). However, the level of *S. mutans* adherence was almost the same among these three alloys (Fig. 8).

There is a possibility that the composition of the pellicle proteins on the alloys may be different, because they had different isoelectric points. The amino acid composition of denture pellicles was clearly different from those of dental pellicles⁶⁹). Therefore, the biofilm form on the different pellicles might be different for different types of samples. In addition, it has been suggested that the adhesion of bacteria is affected by various factors, such as the differences in the charge of the surface layer of the bacteria⁷⁰), van der Waals and electrostatic forces, hydrophobicity and the presence of lectin ligands⁷¹). Another study will be necessary to understand the reasons why *S. mutans* adhered more to Co-Cr than the other alloys.

The results of this study showed that a lot of denture biofilm was attached to the framework alloys. Yamane *et al.* reported²³) that the inter-material differences in the amount of bacteria were smaller than the inter-individual differences in saliva by an *in vivo* study. However, our results showed that there were significant inter-material differences among the framework alloys. This discrepancy might be explained by the differences in experiment models, the kinds of alloy samples and the maturation status of the biofilms. A further *in vivo* study would be needed to clarify the differences among the framework alloys.

CONCLUSION

In conclusion, we made the following findings:

1. The quantitative analysis revealed that the amount of biofilm with total bacteria from saliva or *S. mutans* or *C. albicans* formed on the CP Ti and PGA was almost the same or higher than that formed on the resin surface, while that form on the Ag-Pd-Au was lower than that on the resin.
2. The qualitative analysis of the biofilms by T-RFLP suggested that the bacterial components in the biofilms were diverse, and varied widely among the framework alloys.
3. Oral microorganisms associated with caries,

periodontal disease, aspiration pneumonia and systemic diseases were contained in the biofilms adhered to the framework alloys and resin.

4. It was suggested that some pathogenic bacteria adhered selectively to each alloy.

Based on these results, the quantitative and qualitative properties of the biofilms adhered on the framework alloys were determined.

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