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Immobilization of Octadecyl Ammonium Chloride on the Surface of Titanium and Its Effect on Microbial Colonization *In Vitro*

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The aim of our study was twofold: to immobilize an organosilicon quaternary ammonium salt (3-(trimethoxysilyl)-propyldimethyl-octadecyl ammonium chloride, Si-QAC) on the surface of pure titanium and to investigate the antimicrobial activity of Si-QAC-immobilized titanium against microbial adherence and biofilm formation. The results of ToF-SIMS analysis of Si-QAC-titanium suggested the possibility of immobilizing Si-QAC on titanium surface through Ti-O-Si coupling, and that Si-QAC treatment significantly reduced both the adherence and colonization of *Candida albicans* and *Streptococcus mutans* isolates. The antimicrobial activity was achieved through at least two mechanisms: the first was attributed to the octadecyl alkyl chain which inhibited initial adherence, and the second was attributed to the quaternary ammonium salt which killed initial adherent cells as well as retarded or inhibited subsequent microbial growth. Further, thermocycling did not significantly reduce the antimicrobial activity of Si-QAC-titanium, and no significant cytotoxicity of Si-QAC-titanium was observed in either cell viability test or proinflammatory cytokine production test using human gingival fibroblasts. These results, taken together, favorably suggested that Si-QAC treatment would be a helpful means to inhibit dental plaque or denture plaque formation.

Key words: Octadecyl ammonium chloride, *Streptococcus mutans*, *Candida albicans*, Titanium, Adherence

INTRODUCTION

The applications of titanium for medical and dental purposes have been dramatically increased due to its high resistance to corrosion − which contributes to its excellent biocompatibility, high strength-to-weight ratio, and low modulus of elasticity[1,2]. Commercially pure titanium and its alloys are frequently used for dental implants. More recently, they are also used for crowns, bridges, and partial or complete denture frameworks[3]. This wide range of applications has been attributed to the improvement of casting techniques involving various fields and disciplines[4−9].

Clinically, one of the most serious causes of the failure of implants, crowns and/or bridges is reportedly due to infectious diseases, such as periimplantitis or secondary caries of the abutment teeth. According to a recent review[10], implant failures are divided into early/late and non-infectious (overload)/infectious (periimplantitis) failures. The review of Goodacre *et al.*[11], which covered a literature search of 162 records and studies for the last 50 years, showed that the most commonly reported complication for fixed prostheses was caries (18% of a total of 3360 abutment teeth). On the other hand, denture plaque − which accumulates on denture fitting surfaces − is reported to act as an infection reservoir in *Candida*-associated denture stomatitis (synchronous chronic atrophic candidosis)[12]. It is well known that denture stomatitis is accompanied by acute superficial candidosis, including angular cheilitis, glossitis, oral, oropharyngeal and esophagopharyngeal thrush[13]. It is suggested that the latter lesions can be a source of infection for other forms of oral candidosis in AIDS patients[14].

An organosilicon quaternary compound, 3-(trimethoxysilyl)-propyldimethyl-octadecyl ammonium chloride (Si-QAC), is used as a coupling agent in insoluble polymer contact disinfectants. It has broad-spectrum antimicrobial activity against both Gram-positive and -negative bacteria, fungi, and yeasts[15]. This compound binds chemically to a variety of substrates, making it a nonleachable agent[15]. As such, its antimicrobial activity extends to a diverse range of applications by binding to fibers, fabrics, carpets, and socks (to control odor-causing bacteria). More recently, Gottenbos *et al.* (2002)[16] immobilized Si-QAC on silicon rubber material and showed the efficacy of immobilized antimicrobial agents *in vivo* and *in vitro*. However, little information is available on the antimicrobial treatment of titanium surface − particularly against colonization of oral microorganisms, such as *Streptococcus mutans* (which is widely accepted as a cariogenic pathogen)

and the *Candida* species (which is known as a major cause of opportunistic fungal infections).

Against this background, the aims of our study were to: i) analyze the immobilization of Si-QAC on the surface of pure titanium by means of time-of-flight secondary ion mass spectrometry (ToF-SIMS); ii) investigate the antimicrobial activity of Si-QAC-immobilized titanium (Si-QAC-titan) against microbial adherence and colonization; iii) determine the roles of alkyl chain and quaternary ammonium salt of Si-QAC in the antimicrobial activity; iv) examine the effect of thermocycling on the antimicrobial activity of Si-QAC-titan; and finally v) examine the cytotoxicity of immobilized Si-QAC against human gingival fibroblasts.

MATERIALS AND METHODS

Preparation of specimens

Commercially pure wrought titanium (cp-titan) plates (JIS, Japan Industrial Specification H 4600, 99.9 mass% titanium, 10×10×0.7 mm) were used as the untreated control as well as the substrate material for surface modification. They were finished by wet grinding with 1200 grit SiC paper, then polished using 0.3 μm alumina, and finally cleaned with acetone and ethanol in an ultrasonic bath for 30 minutes respectively.

Cobalt-chromium (Co-Cr), silver-palladium-copper-gold alloy (Au-Ag-Pd), and acrylic resin (AR) were fabricated according to the manufacturers' instructions. Likewise, these specimens were finished by wet grinding with 1200 grit SiC paper, polished using 0.3 μm alumina, and finally cleaned with acetone and ethanol in an ultrasonic bath for 30 minutes respectively. Six specimens of each material were used as the substrate to compare microbial adherence with that on uncoated titanium.

Si-QAC treatment

Octadecyldimethyl(3-trimethoxysilylpropyl)ammonium chloride (Si-QAC) solution (60% in methanol; GELEST, Inc., Morrisville, PA, USA) was diluted with Millipore-Q water to a final concentration of 1, 2, and 5% (vol/vol). Specimens of cp-titan were immersed in 1, 2, and 5% of octadecyldimethyl(3-trimethoxysilylpropyl)ammonium chloride (Si-QAC) solution for 15, 30, 60, and 120 minutes at room temperature (25℃). After the treatment, specimens were washed with sterile distilled water and then with methanol in an ultrasonic bath to remove any excess silane solution. Changes in surface hydrophobicity of the substrates were assessed through Young's contact angles measured against distilled water as a sensing liquid. The mean contact angle ± SD was calculated from six replicated samples.

To examine the contribution of alkyl chain and quaternary ammonium salt of Si-QAC to the antimicrobial activity, cp-titan specimens were treated with 5% (vol/vol) octadecyltrimethoxysilane (C18-titan) (GELEST, Inc., Morrisville, PA, USA) and 5% (vol/vol) γ-aminopropyltriethoxysilane (γ-AMP-titan) (Sigma-Aldrich Japan Co. Inc., Tokyo, Japan) in absolute acetone at room temperature, and washed with acetone ultrasonically to remove any excess silane solution.

Microorganisms and culture condition

Candida albicans GDH 16, GDH 18, GDH 19, *Candida glabrata* GDH 1407 and *Candida tropicalis* GDH 1362, oral isolates obtained from the routine microbiology services of the Glasgow Dental Hospital and School, were used in this study. *C. albicans* IFO 1385, purchased from the Institute for Fermentation, Osaka, Japan, was used as the reference strain. All yeasts were identified using the API 20C system (API Products, Biomeroux, Lyon, France) and the "germ tube" test[17]. A loopful of the yeast was inoculated in yeast nitrogen base medium (Difco, Detroit, USA) containing 250 mM glucose and grown aerobically at 37℃[18]. After overnight culture, the yeast was harvested in the late exponential growth phase, washed twice with sterile distilled water, and resuspended to the final concentration (1×10^5 or 1×10^7 cells/ml) using haemocytometeric counts[18,19].

Streptococcus mutans NCTC 10449 and OMZ 175, provided by the Department of Oral Microbiology, Hiroshima University, were used in the study. These microorganisms were cultured and prepared for experiments according to the method described by Satou *et al.*[20]. Briefly, all isolates were grown in trypticase soy broth (Difco) supplemented with 0.5% yeast extract (Difco) (TSBY). The cells were harvested during the exponential growth phase by centrifugation at 1000 xg, washed twice with sterile distilled water, and resuspended. The cell suspensions were subjected to a low-intensity ultrasonic device to disperse bacterial aggregates[20]. Optical densities (OD) of the suspensions were measured in a 1.0-ml cuvette with a 1-cm light path, and the suspensions were adjusted to give an OD_{660} of 0.3 (corresponding to 3.0×10^8 cfu/ml) or an OD_{660} of 1.0 (corresponding to 1.0×10^{10} cfu/ml) for both isolates of *S. mutans*[21].

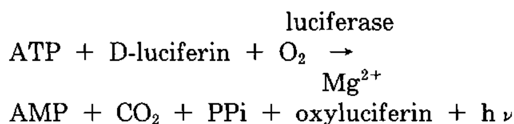
Time-of-flight secondary ion mass spectrometry (ToF-SIMS) analysis of Si-QAC-titan surface

Positive and negative ToF-SIMS analyses were performed using a TRIFT II spectrometer (Ulvac-Phi Inc., Chigasaki, Japan). For spectrum acquisition, the sample was bombarded with pulsed primary $^{69}Ga^+$ ions (15 keV, 3-4 nA dc, pulse length <1 ns when bunched (22 ns unbunched), and repetition rate 5.8 or 8.2 kHz). Analysis area was a square of 100×100 μm. With a data acquisition time of 5 minutes, total ion fluence was approximately $10^{12}-10^{13}$ ions/

cm^2, which ensures static conditions. With these analytical conditions, mass resolution was about 5000 m/Dm at 29 m/z. This highly facilitated the assignment of ions having the same nominal mass but different compositions, for example, hydrocarbons, oxygenated nitrogen-containing fragments, *etc.* To obtain the standard profile of Si-QAC itself, Si-QAC solution was dropped directly on an aluminum foil and the ToF-SIMS analysis performed thereafter.

For the spectra, the secondary ions were accelerated to F3 keV by applying a bias to the sample. Spreading of the initial energies of the secondary ions was compensated by deflection in three electrostatic analyzers. To increase the detection efficiency for high-mass ions, a post acceleration of 5 keV (strikethrough) was applied at the entrance of the detector. No charge compensation was necessary to perform these measurements.

Adherence assay
Adherence assay was conducted as follows. Specimens of cp-titan and Si-QAC-titan were placed in wells of Multiwell tissue culture plates (Nunclon[R] Delta, Nunc, Kamstrup, Denmark.). Two mL of yeast (1×10^7 cells/mL) or streptococcal (1×10^{10} cells/mL) suspension was inoculated on the surface of each specimen, and the whole assembly was incubated at 37℃ for two hours. After which, each specimen was carefully removed and washed thoroughly by rinsing three times for a total of 60 seconds with distilled water to remove loosely adherent organisms. Then, the washed samples were immersed in 1.0 mL of the reagent containing benzalkonium − which extracts intracellular adenosine triphosphate (ATP)[22], and allowed to react for 15 minutes at room temperature[19]. The resultant reagent solution was analyzed by a bioluminescent ATP measuring kit (CellTiter-Glo[TM] Luminescent Cell Viability Assay, Promega KK, Tokyo, Japan) which quantified the ATP content based on the firefly luciferase-luciferin system (The Reporter Microplate Luminometer, Turner Biosystems, Inc., Sunnyvale, Ca, USA), as shown in the following formula[23].



$$\text{ATP} + \text{D-luciferin} + O_2 \xrightarrow[Mg^{2+}]{\text{luciferase}} \text{AMP} + CO_2 + \text{PPi} + \text{oxyluciferin} + h\nu$$

Adherent yeasts on cp-titan and Si-QAC-titan were fixed with 2.5% glutaraldehyde at 4℃ for 24 hours, stained with 1% acridine orange, and viewed with confocal fluorescence microscope (model LSM410, Carl Zeiss, Inc., Germany).

To examine the contribution of alkyl chain and/or positively charged amino group on the initial adherence of the organisms, C18- and γ-AMP-titan were also used as substrates.

The assays were carried out on two independent occasions, with quadruplicated samples on each occasion. All numerical data obtained were analyzed by analysis of variance (ANOVA) and Tukey's multiple range test at 1% significance level.

Saliva and serum
Pooled unstimulated whole saliva was collected from five healthy candidates and clarified, according to the method of Cannon *et al.*[24] with modification, by centrifugation at 12,000 xg for 15 minutes at 4℃. Human serum was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Whole saliva and serum were stored at −25℃ before use[19,25].

Colonization assay
Colonization assay was conducted as follows[19,23,25]. Specimens of cp-titan and Si-QAC-titan were coated with saliva or serum by placing them in wells of Multiwell tissue culture plates (Nunclon[R] Delta, Nunc, Kamstrup, Denmark), into which was dispensed 500 μL of the protein solution per well, and incubated for one hour at 37℃. Saliva or serum was substituted with an equal volume of sterile distilled water in the control wells. After incubation, the protein solution was aspirated. Fifty μL of yeast (1×10^5 cells/mL) or streptococcal (1×10^8 cells/mL) suspension was inoculated on the surface of each cp-titan or Si-QAC-titan specimen, and the whole assembly was incubated at 37℃ for two hours to promote yeast adherence and colonization. Subsequently, 2.0 mL of Sabouraud broth for yeasts or 2.0 mL of TSBY for streptococci was carefully dispensed into each well, and incubated at 37℃ for 72 hours. After which, each specimen was carefully removed and washed thoroughly by rinsing three times for a total of 60 seconds with distilled water to remove loosely adherent organisms or uncolonized organisms. The ATP content of colonized microbes on each specimen was determined using the ATP measuring apparatus based on the firefly luciferase-luciferin system.

To examine the contribution of alkyl chain and/or positively charged amino group on the growth and subsequent colonization of the organisms, C18- and γ-AMP-titan were also used as substrates.

The assays were carried out on two independent occasions, with quadruplicated samples on each occasion. All numerical data obtained were analyzed by analysis of variance (ANOVA) and Tukey's multiple range test at 1% significance level.

Thermal cycling
Specimens of Si-QAC-titan were thermocycled between 4℃ and 70℃ with an immersion time of 60 seconds in each bath, and the antimicrobial assay was carried out after thermal cycling 0, 1250, 2500, 5000, 10000, and 20000 times. Each specimen was used for both adherence and colonization assays as described above. The specimen of 20,000-thermocycled

Si-QAC-titan was also subjected to ToF-SIMS analysis to examine the status of Si-QAC immobilization on titanium surface.

Cell culture and cytotoxicity assay
Human gingival fibroblasts (HGFs) were used in the present study. Healthy gingival tissues were obtained during surgery from donors with their informed consent (subject's rights have been protected by the review board of the Graduate School of Biomedical Sciences, Hiroshima University). The gingival specimens with connective tissue were treated with 0.025% trypsin and 0.02% EDTA overnight at 4℃, and HGFs were isolated from the gingival tissue. The cells were inoculated at a density of 5×10^5 cells per 10-cm plastic tissue culture dish and primarily incubated in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Sanko Junyaku, Tokyo, Japan) supplemented with 10% fetal calf serum, 250 ng/ml amphotericin B, 100 units/ml penicillin, and 100 μg/ml streptomycin, at 37℃ in a humidified 5% CO_2/95% air incubator. At 80% confluence, the cells were harvested with calcium- and magnesium-free phosphate-buffered saline (PBS) containing 0.1% trypsin and 0.1% ethylenediaminetetraacetic acid. The cells were seeded at 1.0×10^4 cells per well in 24-tissue culture plate.

After confluence, each well was inserted with an innerwell (Netwell™, Corning Coster, MA, USA) into which a cp-titan or Si-QAC-titan specimen was immersed, and incubated for 48 hours at 37℃ in a humidified 5% CO_2/95% air incubator. Control well was inserted with Netwell without titanium sample (w/o titanium). After 48 hours' incubation, the cells were gently washed with PBS twice, and the number of vital cells in each well was quantified using an aqueous soluble tetrazolium/formazan assay (MTS assay, Promega, Madison, WI, USA). Protocol for the MTS assay was performed accordingly.

To determine the effects of cp-titan and Si-QAC-titan on HGF secretion of proinflammatory cytokines, *i.e.*, IL-1β, IL-6, IL-8, and tumor necrosis factor alpha (TNF-α), 200 μL of 24-hour incubated culture medium was collected and the supernatants used for enzyme-linked immunosorbent assay (ELISA). The ELISA kit used was Cytoscreen (Bio Source International, Camarillo, CA) for IL-1β, IL-6, IL-8, and TNF-α. To ensure host-independent reproducibility of data, each experiment was repeated at least on two occasions with HGFs derived from different donors.

RESULTS

Contact angle measurements
To preliminarily determine the optimum condition for surface treatment of cp-titan using Si-QAC, contact angle measurements were employed. Young's contact angle on each specimen was dependent on two factors: duration of Si-QAC treatment and concentration of Si-QAC solution (Fig. 1). When treated with 1% Si-QAC solution, Young's contact angle increased initially and then plateaued at 30-minute immersion with $81.8\pm2.6°$. Similarly, Young's contact angle of Si-QAC-titan reached $83.9\pm5.0°$ in 2% solution and $85.5\pm1.9°$ in 5% solution respectively at about 60-minute treatment, and then plateaued. Based upon these results, 5% solution and 60-minute immersion period were employed for the antimicrobial treatment of cp-titan hereafter.

ToF-SIMS spectra
Fig. 2(a) displays a typical positive ToF-SIMS spectrum from a sample of Si-QAC solution dropped directly on an aluminum foil. Two predominant peaks at 296 and 340 m/Z were identified as $C_{20}H_{42}N^+$ and $C_{23}H_{50}N^+$ respectively. Fig. 2(b) displays the positive ToF-SIMS spectrum of Si-QAC-titan. Two major peaks were observed at 296 and 340 m/z, which corresponded to $C_{20}H_{42}N^+$ and $C_{23}H_{50}N^+$ respectively. No peaks were identified from untreated cp-titan specimen. At 92.9 m/z, a peak equivalent to Ti-O-Si$^+$ was specifically detected (Fig. 2(d)), which was not observed on cp-titan.

Adherence assay
Yeast adherence to cp-titan was significantly higher than that to Co-Cr, Au-Ag-Pd, or AR (Fig. 3; ANOVA and Tukey's multiple range test, $p<0.01$).

The initial adherence of microorganisms to cp-titan varied according to genus, species, and strain.

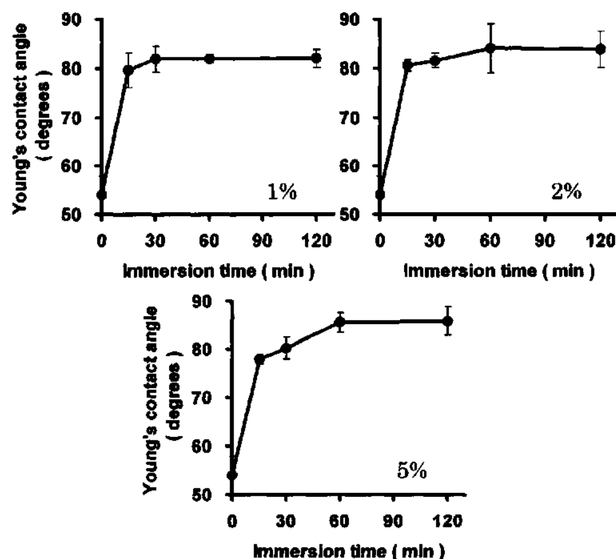


Fig. 1 Relationship between treatment periods and changes in surface hydrophobicity (Young's contact angle) of Si-QAC. Each mean contact angle ± standard deviation was calculated from six measurements.

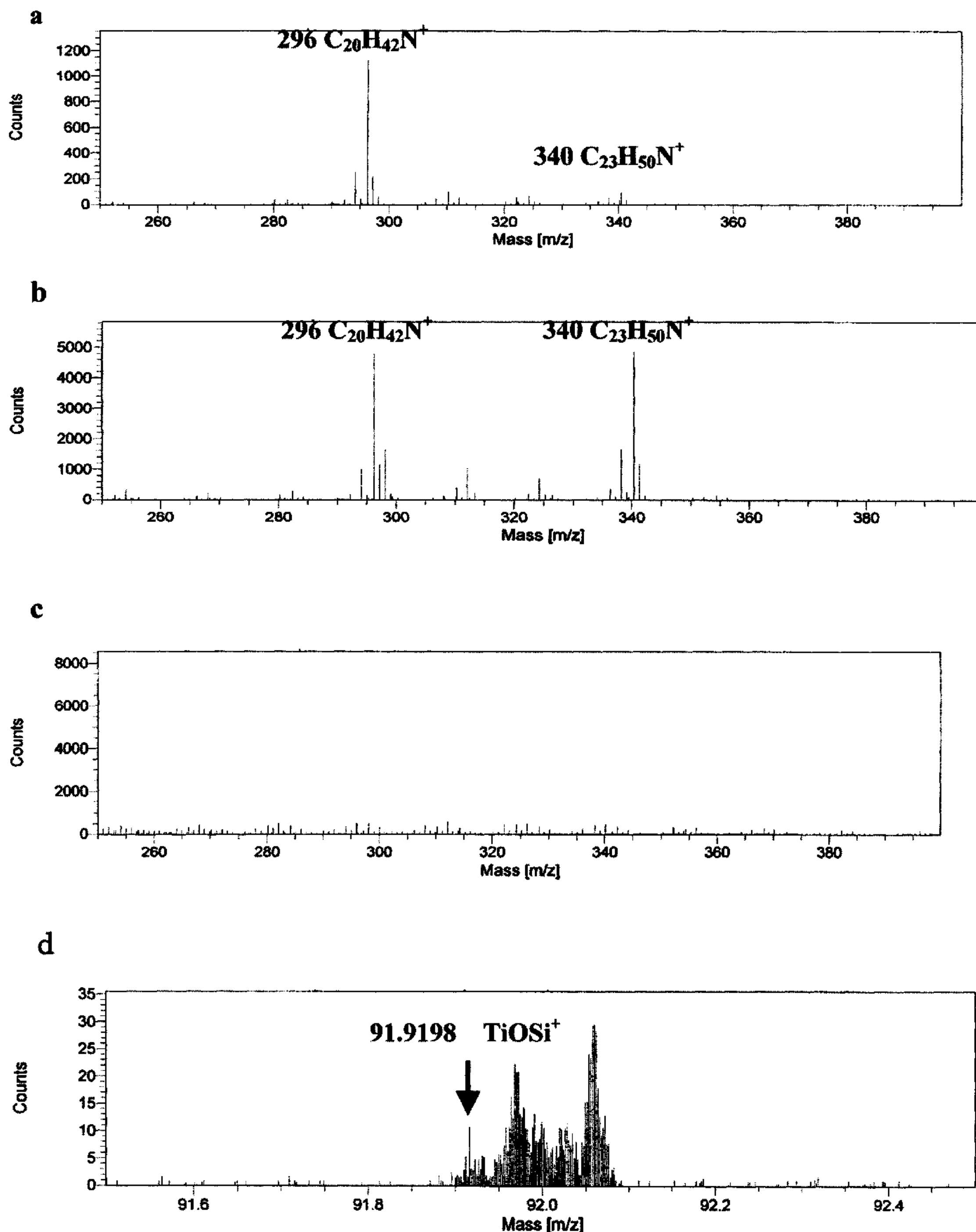


Fig. 2 Typical positive ToF-SIMS spectrum from: (a) a sample of Si-QAC (3-(trimethoxysilyl)-propyldimethyl-octadecyl ammonium chloride) solution dropped on an aluminum foil; (b) Si-QAC-titanium; (c) untreated commercially pure titanium, cp-titan; and (d) Typical positive ToF-SIMS spectrum from a sample of Si-QAC-titan at 92.9 m/z.

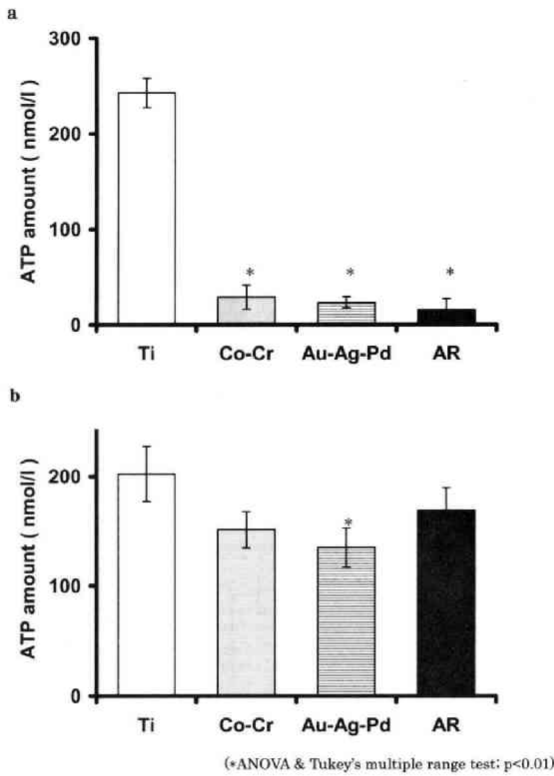


Fig. 3 Adherence to materials used for dentures or crowns/bridges for: (a) *Candida albicans* GDH 16; and (b) *Candida tropicalis* GDH 1362.

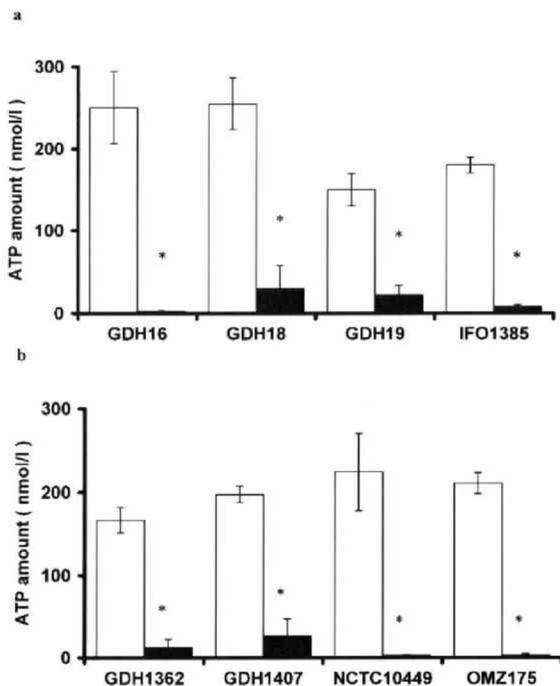
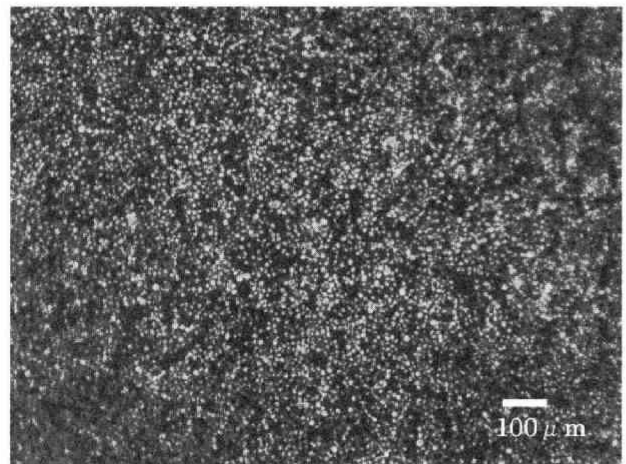
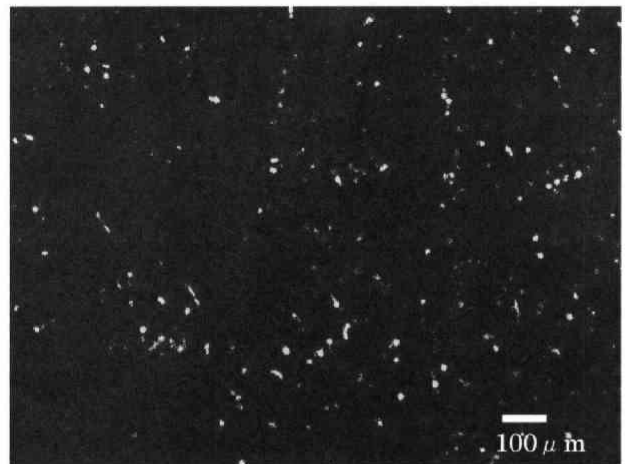


Fig. 4 (a) Adherence of *Candida albicans* isolates to cp-titan (□) and Si-QAC-titan (■); (b) Adherence of *Candida tropicalis* GDH 1362, *Candida glabrata* GDH 1407, and *Streptococcus mutans* NCTC 10449 and OMZ 175 to cp-titan (□) and Si-QAC-titan (■).

However, the adherence was significantly inhibited by Si-QAC treatment in any case (Fig. 4; ANOVA and Tukey's multiple range test, $p<0.01$). In Fig. 5, typical micrograph views of the adherence of *C. albicans* GDH 16 to cp-titan and Si-QAC-titan were shown. For the isolates of *C. albicans* GDH 16 and IFO 1385, they were very sensitive to Si-QAC treatment, and the adherence of these isolates reduced to 1.1% and 4.3% respectively. On the other hand, *C. albicans* GDH 18 and GDH 19 were less sensitive to Si-QAC; nonetheless, the adherence of these isolates were significantly reduced to 14.6% and 11.8% respectively (ANOVA and Tukey's multiple range test, $p<0.01$). The adherence of *C. tropicalis* or *C. glabrata* isolates were also inhibited by Si-QAC treatment to 7.5% and 13.1% respectively (Fig. 4(b); ANOVA and Tukey's



untreated cp-titan



Si-QAC-titan

Fig. 5 A typical micrograph view of the adherence of *Candida albicans* GDH 16 to cp-titan and Si-QAC-titan.

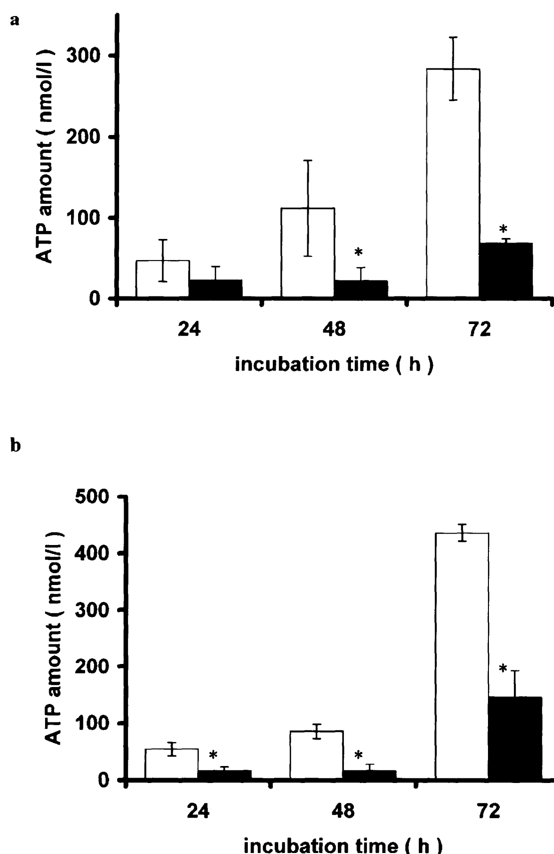


Fig. 6 Colonization of *Candida albicans* GDH 16 (a) and IFO 1385 (b) on cp-titan (□) and Si-QAC-titan (■).

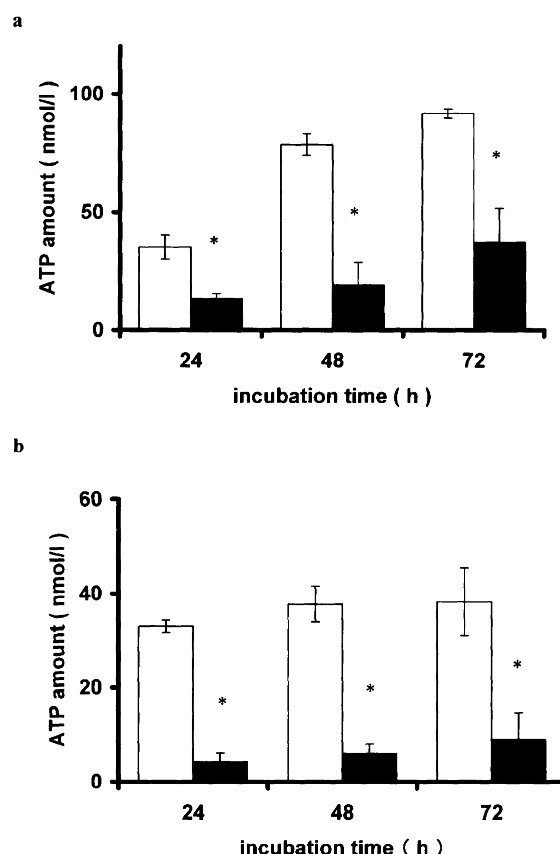


Fig. 7 Colonization of *Streptococcus mutans* NCTC 10449 (a) and OMZ 175 (b) on cp-titan (□) and Si-QAC-titan (■).

multiple range test, $p<0.01$). Similarly, Si-QAC treatment was very effective in inhibiting the adherence of both *S. mutans* isolates: 1.2% for NCTC 10449 and 1.4% for OMZ 175 respectively (Fig. 4(b); ANOVA and Tukey's multiple range test, $p<0.01$).

Colonization assay

In the case of *C. albicans* colonization, the amount of fungi colonized on cp-titan increased during the 72-hour incubation, then plateaued (data not shown), being consistent with our previous results[19]. The colonization of each *C. albicans* isolate was significantly lower on Si-QAC-titan than on cp-titan at each incubation period (Fig. 6; ANOVA and Tukey's multiple range test, $p<0.01$).

Similarly, the colonization of *S. mutans* increased initially, then reached plateau within the 72-hour incubation (data not shown). Si-QAC treatment also significantly reduced the activity of streptococcal colonization at each incubation period (Fig. 7; ANOVA and Tukey's multiple range test, $p<0.01$).

As shown in Figs. 8(a) and (b), the colonization of *C. albicans* or *S. mutans* on saliva-coated and serum-coated Si-QAC-titan was significantly inhibited as compared with that on saliva- or serum-coated cp-titan (ANOVA and Tukey's multiple range test, $p<0.01$).

C18- and AMP-titan

To examine the contribution of alkyl chain and quaternary ammonium salt of Si-QAC to the antimicrobial activity, cp-titan specimens were treated with octadecyltrimethoxysilane (C18-titan; Young's contact angle of the surface was $92.5\pm1.5°$) which possesses long alkyl chain and γ-aminopropyltriethoxysilane (γ-AMP-titan; Young's contact angle of the surface was $64.7\pm1.4°$) which possesses a positively charged primary amino group.

As shown in Fig. 9, Si-QAC-titan was most effective in reducing the initial adherence of *C. albicans* GDH 16, followed by C18-titan. No significant inhibitory effects were observed with γ-AMP-titan (Fig. 9(a)). Moreover, Si-QAC-titan was significantly more effective in reducing microbial adherence than C18-titan (Fig. 9(a); ANOVA and Tukey's multiple range test, $p<0.01$). Against the subsequent colonization, only Si-QAC-titan exhibited the antimicrobial effect (Fig. 9(b); ANOVA and Tukey's

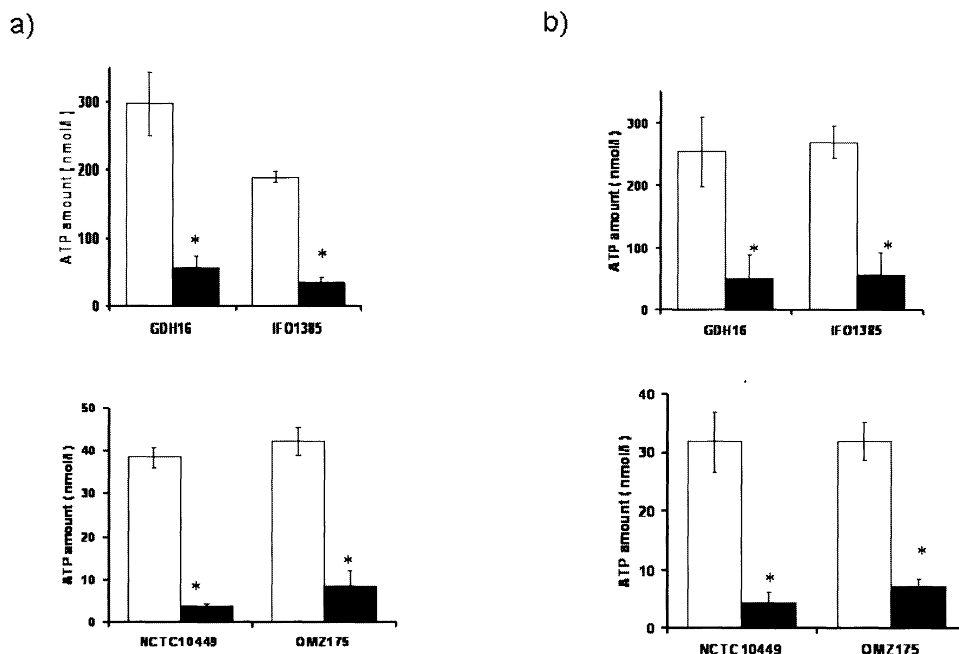


Fig. 8 Biofilm formation of *Candida albicans* (GDH 16 and IFO 1385) and *Streptococcus mutans* (NCTC 10449 and OMZ 175) isolates on: (a) saliva-coated cp-titan (□) and Si-QAC-titan (■); and (b) serum-coated cp-titan (□) and Si-QAC-titan (■).

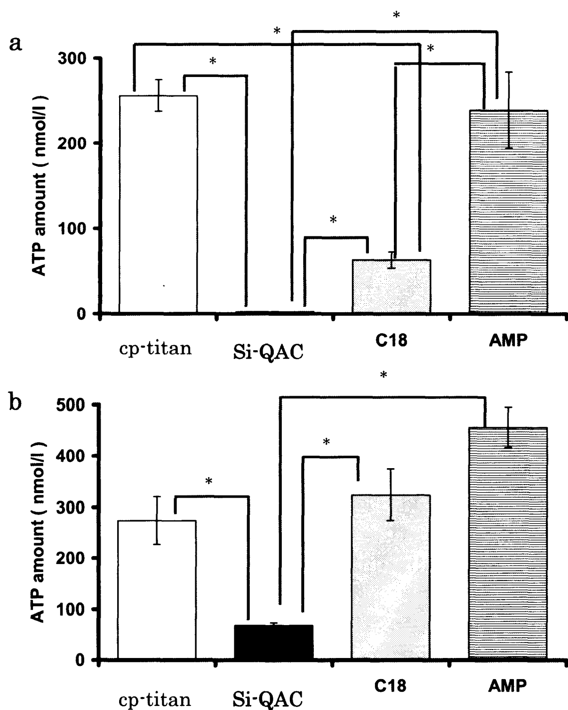


Fig. 9 Initial adherence (a) and subsequent colonization (b) of *Candida albicans* on cp-titan (□), Si-QAC-titan (■), C18-titan (▒) and AMP-titan (▤).

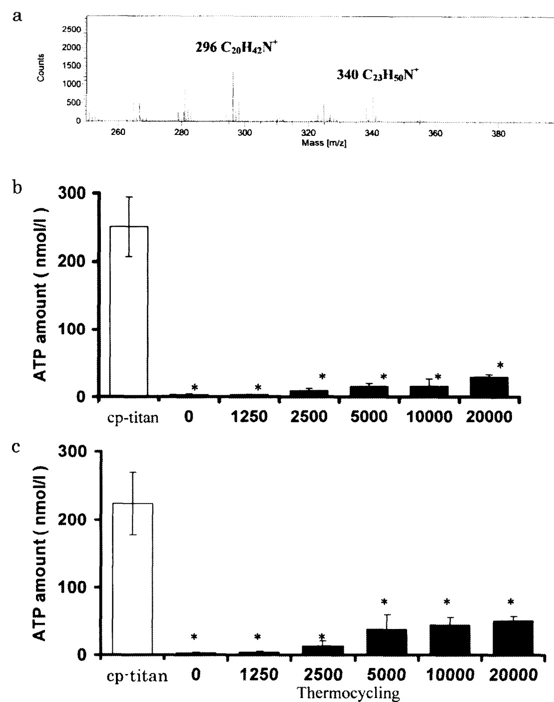


Fig. 10 (a) Positive ToF-SIMS spectrum of 20,000-thermocycled Si-QAC-titan specimen. Antimicrobial effects of thermocycled Si-QAC-titan on: (b) fungal adherence, or (c) streptococcal adherence.

multiple range test, $p<0.01$), and C18-titan showed no antimicrobial effects against microbial colonization.

Thermocycling

The effect of thermocycled Si-QAC-titan on its antimicrobial activity was examined. Fig. 10(a) displays the positive ToF-SIMS spectrum of 20,000-thermocycled specimen of Si-QAC-titan. Two major peaks were observed at 296 and 340 m/z — which corresponded to $C_{20}H_{42}N^+$ and $C_{23}H_{50}N^+$ respectively, confirming that Si-QAC molecules existed on 20,000-thermocycled Si-QAC-titan.

Figs. 10(b) and (c) display the antimicrobial effects of thermocycled Si-QAC-titan on fungal and streptococcal adherence respectively. Although the antimicrobial activity of Si-QAC was reduced in each case as the number of thermal cycles increased, all samples exhibited significant reduction of microbial adherence (ANOVA and Tukey's multiple range test, $p<0.01$).

Cell culture and cytotoxicity assay

Cell viabilities of HGFs incubated for 48 hours without titanium sample (w/o titanium) and with cp-titan or Si-QAC-titan are shown in Fig. 11(a) (ANOVA and Tukey's multiple range test, $p>0.05$). Among the specimens, no significant differences in the viability of HGFs were observed. Similarly, proinflammatory cytokine production by HGFs incubated with Si-QAC-titan were not different from those produced in control wells, *i.e.*, without titanium sample (w/o titanium) and with cp-titan (Fig. 11(b); ANOVA and Tukey's multiple range test, $p>0.05$).

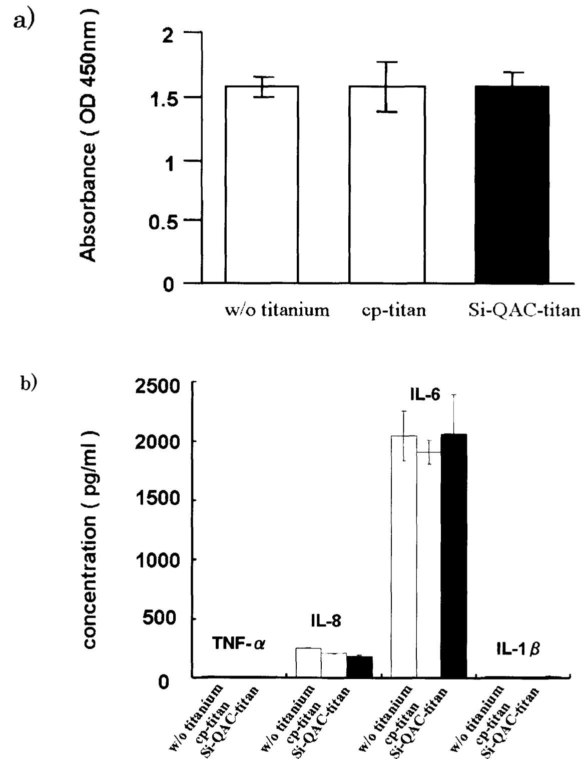


Fig. 11 (a) Cell viability of human gingival fibroblasts (HGFs) incubated for 48 hours without titanium sample (w/o titanium) (□) and with cp-titan (□) or Si-QAC-titan (■); and (b) Proinflammatory cytokine production by HGFs incubated without titanium sample (w/o titanium) (□) and with cp-titan (□) or Si-QAC-titan (■).

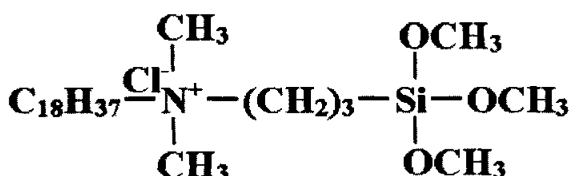
DISCUSSION

Microbial biofilms on prostheses, such as crowns, bridges, dentures, or dental implants are known to cause caries, root caries, periodontitis, and periimplantitis of abutment teeth. In addition, the continuous swallowing or aspiration of microorganisms from dental plaque or denture plaque is considered to expose the patient, particularly an immunocompromised host or medicated elderly, to the risks of unexpected infections[27].

In the preliminary part of this study, the adherence of *C. albicans* to the surface of various dental materials was examined. This was necessary due to gross lack of information on the significance of yeast adherence to titanium, as compared with common dental materials. The adherence of yeast to cp-titan was significantly higher than that to Co-Cr, Au-Ag-Pd, or AR (Fig. 3; ANOVA and Tukey's multiple range test, $p<0.01$), hence confirming the importance of rendering antimicrobial properties to titanium. On this note, we applied an organosilicon quaternary compound — commonly used as a coupling agent — to the surface of cp-titan to control microbial colonization.

Positive ToF-SIMS spectrum of Si-QAC-titan showed two major peaks which corresponded to $C_{20}H_{42}N^+$ and $C_{23}H_{50}N^+$ respectively, implying that Si-QAC molecules existed on Si-QAC-titan. In addition, a peak at 92.9 m/z equivalent to Ti-O-Si^+ was specifically detected (Fig. 2), though no such peak was observed on cp-titan, suggesting the possibility that Si-QAC was immobilized on titanium surface through Ti-O-Si coupling. Recently, Yoshida *et al.* (2002)[28] showed the interaction mechanism between SiO_2 surface and γ-methacryloxypropyltrimethoxysilane (γ-MPTS), and concluded that γ-MPTS chemically bonded to silanol groups at the SiO_2 surface with expulsion of methanol, but not to silyl groups at the Si surface[28]. Similarly, methoxy groups of Si-QAC possibly bonded to Ti-OH groups on the surface of titanium with expulsion of methanol, resulting in the production of Ti-O-Si coupling (Fig. 12).

In the present study, we employed three species



Si-QAC

Octadecyldimethyl (3-trimethoxysilylpropyl) ammonium chloride

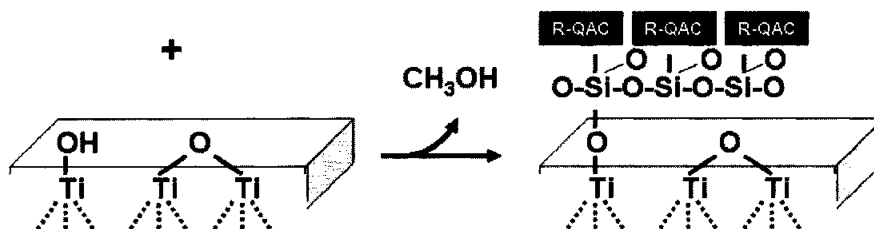


Fig. 12 Schematic presentation of chemical interaction between Si-QAC and titanium.

of *Candida* — which comprised five oral isolates and one type strain — for adherence assay to minimize any interspecies and intraspecies variation in the sensitivity against Si-QAC. *C. albicans*, *C. tropicalis*, and *C. glabrata* are reported to be frequently recovered from the denture surfaces of patients with denture-induced stomatitis[27]. In addition, our previous studies[29−31] indicated that *C. albicans* GDH 16, 18, and 19 had similar capabilities to colonize on acrylic surfaces — with or without the proteinaceous pellicle. However, it must also be mentioned that the three isolates had varied abilities in germ tube formation and hyphal emergence — of which are well known as the virulence factors. GDH 18 showed the highest ability, GDH 19 showed the lowest, and GDH 16 showed an intermediate ability[30,32].

The initial adherence of microorganisms to cp-titan was varied depending on the genus, species, and strain. Nonetheless, in any case, the adherence was significantly inhibited by Si-QAC treatment (Fig. 4; ANOVA and Tukey's multiple range test, $p<0.01$). Against the four isolates of *C. albicans*, the ability to adhere to cp-titan was highest with GDH 16 and GDH 18, followed by IFO 1385 and GDH 19. However, it must be recognized that Si-QAC treatment was significantly effective in reducing the initial adherence of both oral isolates and type strain (Fig. 4; ANOVA and Tukey's multiple range test, $p<0.01$). Hence, we employed GDH 16 and IFO 1385 as the test strains hereafter.

Two isolates of *S. mutans* were employed based on similar reasons mentioned previously. *S. mutans* NCTC 10449 is classified as serotype c, which produces mainly soluble polysaccharides. On the other hand, *S. mutans* OMZ 175 is serotype f, and has been reported to dominantly synthesize insoluble polysaccharides[33]. The ability of the two *S. mutans* isolates to adhere to cp-titan was relatively high when compared with *Candida* isolates. Nonetheless, Si-QAC treatment was significantly effective in reducing the initial adherence of both isolates (Fig. 4; ANOVA and Tukey's multiple range test, $p<0.01$).

The adherence of microorganisms to the surface of dental materials has been thought to be the first step in pathogenesis[26]. Subsequently, during colonization, there will be the growth of adherent cells or the coadhesion of floating or growing cells to adherent ones. In fact, the latter process is just as important too with regard to pathogenesis, colonization, and biofilm formation[19]. However, limited information is available on the interaction between titanium and fungi or streptococci.

The colonization of each *C. albicans* isolate was significantly lower on Si-QAC-titan than on cp-titan (Fig. 6; ANOVA and Tukey's multiple range test, $p<0.01$). Similarly, Si-QAC treatment significantly reduced the activity of streptococcal colonization during the 72-hour incubation period (Fig. 7; ANOVA and Tukey's multiple range test, $p<0.01$).

We further analyzed the influence of saliva or serum pellicle on the inhibitory effect of Si-QAC treatment. This was done because the pellicle, which comprised saliva or serum and which formed on titanium surface, may weaken the inhibitory effect of Si-QAC by modifying the contact between Si-QAC and microorganisms. In addition, the role played by saliva or serum pellicles during the colonization process and the subsequent biofilm formation (whereby multiple layers of cells are embedded within a matrix of extracellular polymeric material) has been re-

ported[19].

The colonization of *C. albicans* or *S. mutans* on saliva-coated and serum-coated Si-QAC-titan was significantly inhibited as compared with that on saliva- or serum-coated cp-titan (ANOVA and Tukey's multiple range test, $p<0.01$). These results, taken together, suggested that Si-QAC treatment of titanium would be an efficacious aid in reducing the colonization of oral pathogens, even though the surface is coated with a proteinaceous layer.

In the present study, we employed ATP quantification of adherent and colonized cells, which measures the number of viable cells[22]. Although Si-QAC essentially killed the microorganisms or retarded their growth, Si-QAC treatment also directly changed the surface hydrophobicity of titanium, thereby modifying adherence or subsequent colonization. Thus, the antimicrobial activity of Si-QAC observed here should be mainly attributed to the killing or antimicrobial effect of the quaternary ammonium salt and the inhibition of initial adherence by the alkyl chain.

To examine the contribution of alkyl chain and quaternary ammonium salt of Si-QAC to the antimicrobial activity, C18- and γ-AMP-titan were prepared. As shown in Fig. 9, Si-QAC-titan was most effective in reducing the initial adherence of *C. albicans* GDH 16, followed by C18-titan, while no significant effect was observed with γ-AMP-titan (Fig. 9; ANOVA and Tukey's multiple range test, $p<0.01$). These results suggested that octadecyl alkyl chain played an important role in reducing initial microbial adherence. As to the observation that Si-QAC-titan was significantly more effective in reducing microbial adherence than C18-titan (Fig. 9; ANOVA and Tukey's multiple range test, $p<0.01$), it could be attributed to the killing effect of the quaternary ammonium salt of Si-QAC. Indeed, only Si-QAC-titan exhibited antimicrobial effect against the colonization of *C. albicans* GDH 16 (Fig. 9; ANOVA and Tukey's multiple range test, $p<0.01$). C18-titan showed no antimicrobial effects against colonization, suggesting that octadecyl alkyl chain itself has little or no effect against microbial growth.

The results, taken together, suggested that Si-QAC-titan exhibited antimicrobial activity through at least two mechanisms. The first was attributed to the octadecyl alkyl chain which inhibited initial adherence, and the second was attributed to the quaternary ammonium salt which killed initial adherent cells and also retarded or inhibited subsequent microbial growth.

In clinical terms, it is very important that Si-QAC treatment ensures prolonged antimicrobial activity. Thus we examined the effect of thermocycled Si-QAC-titan on its antimicrobial activity. The positive ToF-SIMS spectrum of 20,000-thermocycled Si-QAC-titan, with two major peaks at 296 and 340 m/z, confirmed that Si-QAC molecules existed on 20,000-thermocycled Si-QAC-titan (Fig. 10(a)).

Thermocycled Si-QAC-titan still showed significant antimicrobial activity against both fungal and streptococcal adherence, although it must be noted that the antimicrobial activity of Si-QAC was reduced as the number of thermal cycles increased (Figs. 10(b) and (c); ANOVA and Tukey's multiple range test, $p<0.01$). Hence, the thermocycling test illustrated the prolonged antimicrobial activity of Si-QAC-titan.

In addition, no significant cytotoxicity of Si-QAC-titan was observed in either cell viability test or proinflammatory cytokine production test against HGFs (Fig. 11), implying the feasibility of Si-QAC treatment on the surface of prostheses.

Finally, our results, taken together, suggested that Si-QAC treatment of cp-titan effectively reduced both fungal and streptococcal adherence and subsequent colonization — even in the presence of saliva or serum. In addition, the Si-QAC treatment of cp-titan was resistant to thermal cycling and showed no significant cytotoxicity against HGFs, thereby implying the treatment to be a helpful means in inhibiting dental plaque or denture plaque formation.

CONCLUSIONS

In the present study, we examined the immobilization of Si-QAC on the surface of pure titanium by means of time-of-flight secondary ion mass spectrometry (ToF-SIMS), and investigated the antimicrobial activity of Si-QAC-immobilized titanium (Si-QAC-titan) against microbial adherence and colonization. The following results were obtained:

1. The positive ToF-SIMS spectrum of Si-QAC-titan suggested the possibility that Si-QAC was immobilized on titanium surface through Ti-O-Si coupling.
2. Si-QAC treatment significantly reduced the adherence of *C. albicans* and *S. mutans*.
3. Si-QAC treatment of cp-titan effectively reduced the colonization of *C. albicans* and *S. mutans*, even when the surface was coated with a proteinaceous layer like saliva or serum.
4. Si-QAC-titan exhibited antimicrobial activity through at least two mechanisms. The first was attributed to the octadecyl alkyl chain which inhibited initial adherence, and the second was attributed to the quaternary ammonium salt which killed initial adherent cells and also retarded or inhibited subsequent microbial growth.
5. Although the antimicrobial activity of Si-QAC was reduced as the number of thermal cycles increased, 20,000-thermocycled Si-QAC-titan exhibited significant reduction of microbial adherence.
6. No significant cytotoxicity of Si-QAC was observed in both cell viability test and

proinflammatory cytokine production test against human gingival fibroblasts — under the condition that HGFs were isolated from the gingival specimens.

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