Investigation of a novel sterilization method for biofilms formed on titanium surfaces

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The development of effective methods to disinfect biofilms on dental materials is medically important. This study evaluated the bactericidal effects of peroxynitric acid (HOONO₂; PNA) on biofilms formed on titanium surfaces. Streptococcus gordonii was cultured on either machined or rough titanium discs that were then used to evaluate the bactericidal effects of seven reagents, i.e., normal saline, benzalkonium chloride disinfectant solution, chlorhexidine digluconate solution, three concentration types of PNA, and inactivated PNA. Using low concentration of PNA, the bacterial count based on a CFU assay reached an undetectable level within 10 s; this bactericidal effect was the strongest observed for the seven tested reagents. Thus, PNA may be more useful than other disinfectants for sterilizing biofilms on titanium surfaces that have been contaminated with bacteria.

Keywords: Peroxynitric acid (PNA), Sterilization, Peri-implantitis

INTRODUCTION

Prosthodontic treatment with implants is effective for improving occlusion and aesthetic problems; however, despite high implant survival and success rates1,2, there are increasing reports of biological complications, mainly inflammation occurring around the implants. The soft tissue around implants is not as well-sealed3 as that of natural teeth, making the area weak and susceptible to infection. Deep periodontal pockets form due to infection, and these areas turn into anaerobic environments. As bacteria proliferate, bone resorption progresses. In 2008, at the Sixth European Workshop on Periodontology (EWP), peri-implant disease was redefined as follows: peri-implant mucositis was defined as inflammation of the mucosa without any signs of bone loss, and peri-implantitis was defined as inflammation of the mucosa accompanied by supporting bone loss. The incidences of peri-implant mucositis and peri-implantitis are 22 and 43%, respectively4. Thus, while over 90% of implants survive and are functional after treatment, peri-implantitis is still an issue in many cases.

Previous studies of the surface characteristics of titanium, an implant material5-7, have all used a machined surface (MS) in which the implant body was simply polished. However, subsequent improvements to implant systems have resulted in the use of a rough surface (RS), in which fine irregularities are added to the MS via various methods. These surface modifications have substantial effects on calcification, protein absorption, and bacterial and cell adhesion, extension, proliferation, and differentiation8,9. In terms of surface characteristics, the shift from MS to RS increased both the surface area and wettability, making it easier for more osteoprogenitor cells to contact the surface. This change improved implant survival rates for sites with poor bone quality; thus, RS implants have become widely applied.

With a deeper pocket, bacterial adhesion tends to occur in the intricate thread structure of the exposed implant. Once an acquired pellicle has formed on the surface to which glycoproteins have adhered, attached bacteria, such as Streptococcus, settle in the area10 and bind to other bacteria in the oral cavity, forming a large aggregation called a biofilm. Notably, biofilms are highly pathogenic, and inflammatory cytokines produced in response to bacterial irritation destroy peri-implant tissues. Tissue destruction also results from bacterial factors. Common bacterial flora causing peri-implant mucositis and peri-implantitis include Aggregatibacter actinomycetemcomitans and bacteria species belonging to the red complex and orange complex, all of which are associated with periodontal diseases11,12. The state of intraoral bacterial flora prior to implant insertion was reported to affect subsequent biofilm formation on the implant13. Other studies of peri-implantitis sites have indicated that different bacterial types are found around implants and around natural teeth14.

Lang et al. proposed cumulative interceptive supportive therapy (CIST) as a guide for the treatment of peri-implantitis15. This approach involves mechanical cleaning and polishing based on dental plaque accumulation, probing depth, and marginal bone tip changes according to X-ray findings. Contaminants, such
as a biofilm attached to the implant surface and calcified bodies, are then removed to eliminate the substances causing inflammation. If the disease has progressed, bactericidal washing and local or systemic antibacterial drug administration are performed in stages depending on the extent of probing depth and bone resorption. For the most severe cases, surgical treatment is ultimately required. Despite this general treatment policy that cumulatively combines mechanical removal, sterilization treatment, antimicrobial treatment, and surgical treatment, no specific treatment method has been established\textsuperscript{17}. Many methods for mechanically removing contaminants on the implant surface have been reported, including the use of a plastic scaler\textsuperscript{18}, ultrasonic scaler\textsuperscript{19}, rubber cup\textsuperscript{20}, titanium brush\textsuperscript{21}, and powder flow\textsuperscript{22}. Depending on the thread structure, RS properties, and bone loss, it can be difficult to completely remove bacterial biofilms attached to the implant body by mechanical removal alone. Therefore, this study focused on testing potential bactericidal therapies that could be used on biofilms. Since it is difficult to completely sterilize biofilms using existing bactericidal substances, a new bactericidal technique is needed.

The field of plasma medicine, in which atmospheric pressure plasma is applied to human tissues, has garnered increasing attention worldwide. Plasma medicine has applications for disinfection, wound healing, haemostasis, and cancer therapy. However, it would be difficult to use this technology to kill bacteria, which generally exist in liquid, thus, preventing the direct application of plasma for this purpose. To resolve this issue, Ikawa et al. developed the reduced-pH method for extremely effective bactericidal effects\textsuperscript{23}. This technique involves setting the liquid pH to 4.8 or lower so that the decimal reduction time of live bacteria (the D value) reaches 1/100 or lower. The reduced-pH method is based on the principle that superoxide anion radicals (O$_2^{-}\cdot$), a type of reactive oxygen species (ROS) supplied by plasma, are protonated by the acid dissociation equilibrium (pK$_a$ 4.8) in acidic conditions and changed into hydroperoxyl radicals (HOO$^{-}\cdot$), so that HOO$^{-}\cdot$, which is electrically neutral, permeates the cell membrane and applies oxidative stress to the inside of the cell, thereby promoting sterilization\textsuperscript{24}.

In the field of dentistry, studies using a dentin model\textsuperscript{25} and the cavity of a human caries-infected dentin model\textsuperscript{26} have shown that plasma application at low pH makes it possible to effectively sterilize infection-causing microorganisms. However, direct plasma application has various disadvantages, including the requirement for special equipment and the relatively long period of time required (180 s) to reduce bacteria to an undetectable level. Therefore, Tasaki et al. applied plasma-treated water (PTW) to the cavity of a human caries-infected dentin removal model. PTW was prepared outside of the oral cavity, with high concentration of active species, and application of the PTW resulted in reduction of live bacteria to an undetectable level in 10 s\textsuperscript{27}. Additionally, despite a lack of sufficient bactericidal effects when plasma is applied to infected root canal models in which many bacteria are observed within the complex microstructure, a strong bactericidal effect can be achieved using PTW\textsuperscript{28}. Ikawa et al. also demonstrated that PTW exhibits strong sterilizing activity in low pH conditions and that its half-life is strongly temperature-dependent.

On the basis of physicochemical characteristics of PTW, it was revealed that PNA is major active species for sterilization\textsuperscript{29}. PNA is known to dissociate into HOO$^{-}\cdot$ and NO$_2^{-}\cdot$ especially in acidic conditions\textsuperscript{30-32}. The HOO$^{-}\cdot$ that is supplied by this radical dissociation is thought to be the major bactericidal factor in the reduced-pH method. As same as the PTW, PNA dissociates into HOO$^{-}\cdot$ and NO$_2^{-}\cdot$ especially in acidic condition. Superoxide anion radicals (O$_2^{-}\cdot$) are protonated by the acid dissociation equilibrium (pK$_a$ 4.8) in acidic conditions and changed into hydroperoxyl radicals (HOO$^{-}\cdot$), HOO$^{-}\cdot$ easily permeates the cell membrane, because it is electrically neutral, and inside the cell, it chemically modifies amino acid residues to inactivate protein, thereby promoting sterilization. Thus, the plasma application step is no longer necessary, making its clinical application simple compared with previous methods because PNA can be produced by a simple chemical reaction\textsuperscript{33,34}. Although PNA has long been known to a reactive nitrogen species, it has rarely been applied in any fields, and it has not also yet been used for sterilization. In this study, we aimed to develop a novel method for sterilizing biofilms by applying chemically synthesized PNA to titanium surfaces for the treatment of peri-implantitis. We prepared a model of biofilm formation on titanium surfaces and compared the bactericidal effects of PNA solutions and various alternative disinfectants.

### MATERIALS AND METHODS

#### Titanium disc preparation

JIS type 2 pure titanium discs (diameter: 12.0 mm, thickness: 1.5 mm; Sky Blue, Fukuoka, Japan) were subjected to machine polishing to create MS specimens or sandblasting/etching to create RS specimens. Alumina particles with a surface diameter of 50 µm were used for sandblasting the MS specimens. After a thorough washing with distilled water, the discs were soaked for 30 s at 25°C in a 15% hydrofluoric acid aqueous reagent, thoroughly washed again with distilled water, and soaked for 3 min in a solution made from a 1:1 ratio of 95.5% sulfuric acid and 31.3% hydrochloric acid. The specimens were then thoroughly washed with distilled water. Before use in an experiment, the titanium discs were enclosed in a sterilized packet and subjected to gamma ray sterilization. The packets were opened immediately before use.

#### Pellicle formation

Resting saliva was obtained from four healthy volunteers (two men and two women). To remove impurities, the saliva was subjected to filter sterilization (Millex HA 0.45 µM, Merck Millipore, Billerica, MA, USA) according
to a method described by Hirota et al., involving centrifugation at 4°C and 12,000×g for 20 min followed by collection of the resulting supernatant. The sterilized saliva from the four subjects was then mixed and divided into four samples of equal volume. For the experiment, titanium discs were placed in the wells of a 12-well plate (Falcon, Corning, NY, USA), and 2 mL of experimental saliva was added to each well. The specimens were left to react for 30 min at 37°C under a 5% CO₂ atmosphere to facilitate pellicle formation. All operations involving the titanium discs were performed aseptically. The protocol was approved by the Ethics Committee of Kyushu University (Permission No. 22010).

Culture conditions and bacterial strain
Streptococcus gordonii (ATCC 10558) was used for surface adhesion to the titanium discs. A single colony of S. gordonii cultured on blood agar media was seeded in brain heart infusion broth (BHI; Difco, Grand Island, NY, USA) and cultured at 37°C under a 5% CO₂ atmosphere.

Biofilm formation on titanium discs
S. gordonii harvested from culture medium was suspended in BHI (7 mL with 140 µL of 1% glucose). The test saliva was removed from the wells containing the titanium discs with pellicles, 50 µL of BHI bacterial suspension was added, and the samples were left to react for 30 min at 37°C under a 5% CO₂ atmosphere to promote biofilm formation. After biofilms had formed, the titanium discs were transferred from the BHI bacterial suspension to phosphate-buffered saline (PBS), washed gently with new PBS twice, and then placed in new wells.

Preparation of the PNA reagent
The synthesized PNA reagent was mixed by 45 µL of 1.0 mol/L HNO₃ with 60 µL of 6.0% H₂O₂ and was stored before adding 50 µL of 10% NaNO₂ on ice. The PNA concentration of the synthesized reagent was 120 mmol/L.

The synthesized PNA reagent was diluted to prepare PNA test reagents with concentrations of 4.6 mmol/L, 2.3 mmol/L, or 0.92 mmol/L. To prepare PNA test reagents, synthesized PNA reagent was diluted by sodium citrate buffer to keep the pH at 3.2 during bactericidal experiment. Final concentration of citrate buffer was 20 mmol/L. PNA synthesis and dilution were performed directly before the sterilization experiment.

PNA inactivation
A PNA reagent with an initial concentration of 4.6 mmol/L was prepared as described above and maintained at 27°C (room temperature) for 24 h to completely degrade the PNA. This was used as a inactivated PNA reagent. A CFU assay confirmed that this inactivated PNA solution was completely inactive.

Biofilm sterilization experiment
The bactericidal effects against biofilms adhered to titanium discs of seven reagents, i.e., normal saline (NS; Otsuka Pharmaceutical, Tokyo, Japan), 0.025% benzalkonium chloride solution (BZC; Yoshida Pharmaceutical, Tokyo, Japan), 0.2% chlorhexidine digluconate solution (CHX; SIGMA-ALDRICH, Tokyo, Japan), PNA solutions at concentrations of 4.6 mmol/L, 2.3 mmol/L, and 0.92 mmol/L, and a inactivated PNA solution, were evaluated. Nine biofilm adhered titanium discs were prepared for each group, then the titanium discs were placed into separate wells, and 2.0 mL of each reagent was applied to the wells (for 0, 10, 20, or 30 s), followed by the addition of soybean-casein digest broth with lecithin & polysorbate 80 (Nihon Pharmaceutical, Tokyo, Japan) at the same volume (2 mL) to stop the sterilization reaction. Samples were then washed with PBS. Each process was performed statically to prevent detachment of the biofilm. Nine titanium discs per group were placed into 2 mL microtubes containing 900 µL of BHI and mixed in a tube mixer to detach the biofilm from the titanium disc and suspend bacterial cells uniformly. After treatment with each of the reagents, a colony-forming unit (CFU) assay was carried out to assess the bactericidal activity of PNA. Each dilution (100 µL) was spread on an BHI agar plate and incubated for 3 days at 37°C, at which point colonies were counted. The minimum detection value was 10 CFU mL⁻¹. The number of surviving bacteria was determined by the CFU assay, as described above.

Observation of biofilm attachment on titanium surfaces with crystal violet staining
A correlation has been demonstrated between crystal violet pigment absorption and the dried biofilm weight for various types of biofilms[30]. Crystal violet is an alkaline purple pigment with a triphenylmethane structure used to stain the bacterial cell wall. Nine biofilm adhered titanium discs were prepared for then each group, the titanium discs were gently washed twice with PBS, and the resulting supernatant was removed. Without treatment with various reagents, a 0.1% crystal violet reagent was added to each of the titanium wells. After 15 min of staining, the samples were lightly washed twice with PBS to remove any excess dye and dried with air flow. For comparison, the same processes were performed on titanium discs after PNA treatment. This method made it possible to assess whether physical operations caused biofilm detachment from the titanium surface.

Observation of S. gordonii adhesion by scanning electron microscopy
The nine titanium discs with biofilms per group were treated with NS, 0.025% BZC, 0.2% CHX, or 4.6, 2.3, or 0.92 mmol/L PNA solutions for 30 s. They were then soaked in 2 mL of a fixing reagent (2.5% glutaraldehyde and 4% formalin) and incubated for 30 min at 4°C to fix the biofilms. Samples were subsequently soaked in 50% ethanol, 70% ethanol, 100% ethanol, and 100% ethanol/tert-butyl alcohol at equal volumes, and tert-butyl alcohol for 5 min. The tert-butyl alcohol was
replaced twice, and the specimens were then frozen at 4°C before sublimating the tert-butyl alcohol by freeze-drying (JFD-300, JEOL, Tokyo, Japan). A specimen preparation electron microscope (MSP-18, VACUUM DEVICE, Ibaraki, Japan) was used for electrical conduction. An ultra-high-reagent field emission scanning electron microscope (FE-SEM; SU8000, Hitachi High-Technologies, Tokyo, Japan) was used to observe S. gordonii surface attachment on each titanium disc with an acceleration voltage of 1.0 kV.

Statistical analyses
Bacterial counts were converted to logarithmic values. One-way analysis of variance (ANOVA) and post-hoc Tukey-Kramer tests were used for statistical analyses. The level of significance was set to $p=0.05$.

RESULTS

Biofilm attachment before and after treatment with various reagents
After crystal violet staining was performed on the uncultured raw titanium discs, no culture was not stained with crystal violet (Fig. 1a). On the other hand, biofilm formation was observed macroscopically on the whole surface of the titanium discs in the post-culture MS group (Fig. 1b). Visual observation of the surface biofilms in the MS group after treatment with each of the reagents (NS, 0.025% BZC, 0.2% CHX, 4.6 mmol/L PNA solutions and inactivated PNA solution) did not indicate substantial decreases in the amount, suggesting that substantial biofilm attachment remained despite treatment (Figs. 1c–g). Similar results were obtained for the titanium discs in the RS group (Figs. 2a–g).

SEM was performed to compare the biofilm attachment before and 24 h after S. gordonii culture on surfaces of titanium discs in the MS and RS groups. Concentric line structure was observed in the MS group prior to culture (Figs. 3a, b). In the RS group, the metallic lustre characteristic of the machined titanium surface was absent (Figs. 4a) and irregular waves were observed (Figs. 4b). A milky white biofilm was attached to each of the titanium discs in the post-culture MS and RS groups (Figs. 3c, 4c), and SEM images confirmed the presence of second corn cob-shaped Streptococci in these biofilms (Figs. 3d, 4d). We were thus able to confirm observable biofilm formation after 24 h of S. gordonii culture (Figs. 3c, d and Figs. 4c, d). SEM observations confirmed the presence of bacteria on the post-disinfected discs with any of the tested reagents. Similar results were obtained for the titanium discs in the MS and RS groups (Figs. 5a–e). This demonstrated that little physical biofilm detachment resulted from the chemical actions of these reagents at the concentrations used in this study. The results of RS and MS experiments are identical.

![Fig. 1 Crystal violet staining of biofilm adhesion on MS titanium discs.](image1)

![Fig. 2 Crystal violet staining of biofilm adhesion on RS titanium discs.](image2)

a: no culture, b: no chemical treatment, c: normal saline, d: benzalkonium chloride disinfectant reagent, e: chlorhexidine digluconate reagent, f: PNA (4.6 mmol/L), g: inactivated PNA.
Fig. 3 Pre-/post-culture biofilm adhesion on MS titanium discs. 
a: pre-culture Ti disc, b: pre-culture, c: post-24 h incubation, d: post-24 h incubation Ti disc. 
(b, d: SEM images of pre-culture and post-24 h incubation. Scale bar: 1 mm at low magnification, 20 µm at secondary electron image).

Fig. 4 Pre-/post-culture biofilm adhesion on RS titanium discs. 
a: pre-incubation Ti disc, b: pre-incubation, c: post-24 h Incubation, d: post-24 h incubation Ti disc 
(b, d: SEM images of pre-culture and post-24 h incubation. Scale bar: 1 mm at low magnification, 20 µm at secondary electron image).

Fig. 5 S. gordonii adhesion on MS titanium discs after treatment with various reagents. 
a: normal saline, b: benzalkonium chloride disinfectant reagent, c: chlorhexidine digluconatereagent, d: PNA (4.6 mmol/L), e: inactivated PNA (a–e: SEM images of post-chemical treatment. scale bar: 20 µm at secondary electron image).

Bactericidal effects of various reagents against S. gordonii biofilms

The time for titanium surface sterilization and viable bacteria counts were measured for titanium discs in the MS and RS groups after treatment with NS, 0.025% BZC, 0.2% CHX, or 4.6 mmol/L PNA solutions. Viable bacteria on the discs in the PNA group (4.6 mmol/L) were reduced to undetectable level after 10 s (Fig. 6),
while in the other tested reagents, hardly any changes were noted, even after 30 s (Fig. 6). The results differed significantly between PNA group at from 10 s to 240 s and the others. And the results differed significantly between CHX group at 120 s and NS, BZC and inactivated PNA groups. Viable bacteria counts in the CHX group at 120 s and NS, BZC began to decrease at 120 s later and reached an undetectable level after 300 s (Fig. 7). The results differed significantly between PNA group at from 10 s to 240 s and the others. The results differed significantly between CHX group at 60 s. Moreover, the results differed significantly between CHX group at after 120 s and NS, BZC and inactivated PNA groups.

**Effect of PNA concentration on bacteria inactivation**

Based on the observed bacteria effect of 4.6 mmol/L PNA on mean bacterial counts for titanium surfaces at each treatment time, these metrics were assessed for three different PNA concentrations using titanium discs in the MS and RS groups. Viable bacteria reduced to undetectable levels in 10 s in two of the PNA groups (4.6 and 2.3 mmol/L) (Figs. 8 and 9). In the lowest PNA concentration of 0.92 mmol/L, the bacteria counts were lower than detection limit (Tukey-Kramer test, \( p < 0.05 \)).
concentration group (0.92 mmol/L), viable bacteria tended to begin to decrease at 30 s later and reached undetectable levels at 240 s. No differences in the effects of these PNA concentrations were noted between the MS and RS groups. The results differed significantly between PNA group at concentration of 0.92 mmol/L and the others at from 10 s to 240 s.

**DISCUSSION**

In this study, we used *S. gordonii*, a Gram-positive facultative anaerobic bacteria that has been detected at high rates in implant components\(^{27}\), to investigate biofilm characteristics on titanium discs. Previous work has shown that *S. gordonii* exhibits early attachment to pellicles that form on the tooth surface. These bacteria settle on glycoproteins and enable initial plaque formation, after which they combine with various other bacteria to form a large aggregation\(^{19}\). Notably, not all bacteria bind strongly to the pellicle. *S. gordonii* is a typical early-colonizing species of bacteria that exhibits strong binding ability in the initial stages of biofilm formation. Bacteria that repeatedly attach by co-agglutination to these early colonizing bacteria are called late-colonizing bacteria. Gram-negative obligate anaerobic bacteria, such as *Fusobacterium nucleatum*, *Prevotella intermedia*, *Tannerella forsythia*, *Porphyromonas gingivalis*, and *A. actinomycetemcomitans*, have been detected in local sites of peri-implantitis\(^{18,29}\). It has been reported that obligate anaerobic bacteria can survive for 2 h in the atmosphere\(^{49}\). Moreover, because these anaerobic bacteria exhibit little resistance to ROS, they are thought to be more sensitive to PNA than *S. gordonii*. Therefore, we expected that PNA concentrations high enough to inactivate *S. gordonii* would also be high enough to ensure sufficient bactericidal effects against the obligate anaerobic bacteria.

Here, our CFU assay results indicated that PNA solutions have strong bactericidal effects, with the ability to reduce bacterial counts to undetectable levels within a short period of time. BZC and CHX, which were used as controls for comparison with the PNA solutions, are used to disinfect not only living organisms but also inanimate objects, such as medical equipment. CHX has a wide spectrum of sterilizing effects, acting against both bacteria and fungi. It has excellent bactericidal power against many types of bacteria, causing minimal irritation of the skin and mucosa and exhibiting sustained effect of drug after the initial disinfection owing to its tissue retentive ability. In our experiment, a significant sterilizing activity of CHX was observed within 300 s. In contrast, BZC exhibited hardly any sterilizing activity, even after 300 s, whereas both 4.6 and 2.3 mmol/L PNA solutions were able to reduce viable bacteria to undetectable levels within 10 s. The lowest tested PNA concentration, 0.92 mmol/L, exhibited similar bactericidal activity to that of CHX. Treatment time should be as short as possible, PNA which achieves sufficient sterilization in 10 s, may be more clinically useful than CHX, which takes 300 s to sterilize. Therefore, our results suggest that, due to the speed and power with which they cause sterilization, PNA reagents could be more useful than other disinfectants for biofilm sterilization.

Although previous studies have demonstrated that plasma application\(^{25,26}\) and plasma processing\(^{27,28}\) exhibit strong sterilizing activity against oral microbes, our study is the first to demonstrate that synthesized PNA exhibits extremely strong bactericidal effects against biofilms. As the mechanism of action for PNA involves sterilization *via* ROS, the probability of resistant bacteria emerging is very low, making it a highly useful approach. Moreover, because there is hardly any pH regulation of bodily fluids with a pH buffering capacity and normal tissues are protected by an external matrix, damage caused by oxidative stress due to PNA is expected to be low, suggesting that the reagent is likely very safe.

The temperature dependence of the half-life of PNA is extremely high\(^{29}\). Despite having a half-life of several tens of seconds on ice, it becomes inactivated within seconds at body temperature. As such, there is minimal residual toxicity. However, one expected disadvantage associated with the application of PNA in the oral cavity is the difficulty in ensuring that the reagent reaches the depths of periodontal pockets and remains for sufficient periods owing to its short half-life. This limitation may be addressed by efficient mechanical cleaning prior to PNA application to expose the sterilization sites, lowering the temperatures of the PNA solution and sterilization site, and making the sterilization site acidic in advance. Owing to its short half-life, it is desirable that PNA is synthesized immediately before use. However, if frozen, long-term storage is possible. Chemical synthesis is less expensive than plasma synthesis. It is known that half-life time of PNA is strongly temperature-dependent and a few seconds at biological condition. Taking chemicals diffusion speed into consideration, we think that PNA is safe for human body, because it is difficult for PNA to reach the lower layer of the stratified squamous epithelium within a few seconds. That dissociation product of PNA is sodium nitrate and it is known to be safe. More studies are needed to apply it to clinical settings, because it is a new sterilization agent. Owing to its potential for large-scale synthesis and strong bactericidal effects, PNA could become highly valuable in the field of medicine. Thus, we anticipate its use as a novel sterilizing agent to replace conventional disinfectants. Therefore, experiments using 3-dimensional cultured skin model are planned to verify the safety of PNA. However, it is likely that the effective concentration of PNA *in vivo* is not the same as this study *in vitro*. Therefore, we need to test the effective clinical concentration of PNA in the future.

**CONCLUSION**

In this study, we prepared biofilm models by culturing *S. gordonii* on machined and rough titanium surfaces.
We observed that PNA exhibits strong bactericidal effects on both biofilm adhesion models.

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