

Original

**Antimicrobial Property of Titanium Plates Treated
with Silica-Bound Protamine**

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Abstract : Antimicrobial activity is necessary for dental prosthetic devices to maintain oral and systemic health in elderly people wearing prostheses. In particular, dental prosthetic devices with antifungal properties are urgently needed to prevent aspiration pneumonia. However, practical application methods to deliver antimicrobial properties to dental prosthetic devices have not yet been established. Therefore, in this study we aimed to fix protamine on titanium plates treated with silica coating using a Silano-Pen, and to evaluate the antimicrobial properties of the titanium plates against *Candida albicans*. Strong antifungal properties were obtained by soaking titanium plates in an aqueous protamine solution after silica coating treatment. Since this brand-new method is simple, its practical application is expected in the near future.

Key words : antimicrobial, protamine, titanium, silica coating

Introduction

According to the demographic statistics of the Japanese Ministry of Health, Labour and Welfare, 35,740 people died with aspiration pneumonitis in 2017, ranking it as the seventh leading cause of death in Japan¹⁾. Oral care is effective in preventing this disease;²⁾ however, elderly people often wear dental prosthetic devices (dentures), which can provide a scaffold for microbial growth. To prevent this, it is necessary to keep the denture clean, but it is difficult for many patients to obtain frequent professional care. Therefore, it is desirable that the dentures themselves have antimicrobial properties. However, antimicrobial dental materials are not yet available, and when antimicrobial substances are incorporated into dental materials, side effects and the development of antimicrobial resistance may arise. Numerous attempts have been made to solve these problems, such as mixing with antibacterial inorganic powders³⁻⁶⁾ and organic liquids⁷⁻¹⁰⁾. But these attempts have not been successful practically.

One promising approach is to adsorb an antimicrobial protein contained in saliva onto the surface of the dental prosthetic devices to obtain a persistent effect. Because such peptides are very expensive, we searched for commercially available antimicrobial peptides that might have a similar effect. Protamine, a natural food preservative, has been used by food suppliers since the

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1980s because of its strong antimicrobial effects¹¹⁾. Protamine is extremely heat stable and has a preservative effect in neutral and alkaline foods.

In the dental field, Miura *et al*^{12,13)} reported the efficacy of protamine against periodontal pathogenic bacteria and modified its antifungal activities with polymethyl methacrylate. Maruha Nichiro Corp.^{14,15)} found that hydrolyzed protamine acquires antifungal activity. Matsuda found that protamine and a silica aqueous solution precipitates immediately after mixing and the powder shows antibacterial and antifungal effects¹⁶⁾. We suspected that attaching protamine to the silica surface might produce a similar change in the peptide structure to that induced by hydrolysis.

In dentistry, silicatization has been used for the pretreatment of adhesion enhancement materials for crowns and bridges since the 1980s¹⁷⁾ (e.g., the Silicoater System; Kulzer & Co. GmbH, Hanau, Germany, and the Silano-Pen; Bredent, Senden, Germany). Among the materials for dental prosthetic devices, titanium has recently become the most popular because of its excellent biocompatibility and mechanical properties and it is now widely applied to many kinds of devices, including removable dentures.

Therefore, in this study, we firstly aimed to perform silicatization onto a titanium surface using the Silano-Pen because it is a handy and clinically acceptable device. Additionally, we verified spectroscopically that protamine could adsorb onto the silica adhering to the titanium surface, and then examined its antimicrobial properties. Additionally, because silica treatment is not generally used on acrylic resin denture bases, we describe a silica treatment method that does not cause damage to the denture.

Materials and methods

Reflection-absorption spectroscopy (RAS) and micro Fourier-transform infrared (FTIR) analysis of titanium surfaces treated with silicatization

Sample plates with a thickness of 1.3 mm were prepared from a pure titanium rod (JIS class II) by slicing with a slow saw cutter (Buehler Isomet[®], Lake Bluff, IL, USA). Each plate was polished with silicon carbide paper #800 and a diamond paste. Next, the titanium plates were treated with a Silano-Pen (Fig. 1) following the manufacturer's instructions. The plates were then dipped into an aqueous 4% protamine solution (Maruha Nichiro, Tokyo, Japan).

To analyze surface absorption, RAS-FTIR analysis was performed (PR-510, JASCO, Tokyo, Japan) with the angle of the plate being 70°. An RAS-FTIR spectrum was obtained and, after the plate was washed with water to remove soluble material, the spectrum was obtained again. The sample surface was then scraped to collect a specimen for direct micro-FTIR analysis using a JASCO IRF-3000 instrument. The material was mixed with potassium bromide powder and pressed to make a thin pellet.

Antimicrobial testing of the titanium plates

A titanium plate with a thickness of 2 mm was cut to 50 × 50 mm using a wire-type electrical discharge machining device (FANAC RoboCut α -0iA, Yamanashi, Japan) and the plate surface

was treated as described above. An antimicrobial test was performed according to ISO 22196 (JIS Z2801) "Measurement of antibacterial activity on plastics and other non-porous surfaces"^{18,19)}. Culture fluid (0.1 ml) containing *Staphylococcus aureus* (NBRC12732) at 2.6×10^5 colony-forming units (CFU)/ml, *Escherichia coli* (NBRC3972) at 1.0×10^5 CFU/ml, or *Candida albicans* (NBRC1594) at 3.7×10^4 CFU/ml were spotted onto each sample, and a polypropylene film covered the sample (N=3). The samples were incubated at 35°C for 3 hr for bacteria or 24 hr for *C. albicans*. A soybean-casein digest agar with lecithin and polysorbate agent was used to wash out the microbes, and a standard agar medium was added and incubated at 37°C for 24 hr. After this, the colonies were counted.

Denture surface treatment trial

An acrylic resin plate was prepared using auto cure type resin (GC, Unifast II Tokyo, Japan) formed between glass plates. Information on the temperature at the time of treatment with a Silano-Pen was collected using a FLIR ONE Pro (FLIR Systems Inc., Portland, OR, USA) thermal imaging camera.

Results

Surface analysis

Figure 2 shows the RAS-FTIR spectra of the protamine-treated polished titanium plates before and after the water wash. Amide peaks disappeared after the wash. RAS-FTIR spectroscopy created a strong-intensity enhancement on adsorbed polished metal²⁰⁾. Therefore, this finding suggests that protamine was attached to the metal surface before the wash, and disappeared from the metal surface after the wash. The unaffected Si-O band at around $1,200 \text{ cm}^{-1}$ indicates that the silica remained firmly attached to the surface.

Figure 3 shows an image of how the sample surface was scraped and mixed with potassium bromide (left), a microscopic view of the sample (middle), and the micro-FTIR spectrum (right). The scraped surface powder contained clear amide peaks in the FTIR spectroscopy, indicating that protamine was present in the silica powder scraped from the surface. When combined with the data in Figure 2, these findings suggest that protamine did not attach directly to the metal surface but did adsorb onto the surface-attached silica.

Antimicrobial tests

Figure 4 shows the result of ISO 22196 (JIS Z2801) antimicrobial tests, with the mean number of microbes plotted on a logarithmic scale. The initial value shows the number of microbes collected and counted immediately after inoculation. Two kinds of control data were obtained: for film (Control in the figure) and the untreated titanium (Ti in the figure).

The colony count was markedly lower in treated specimens. According to the test standard, a decrease of 2 log-units after 24 hr is an antimicrobial effect; our treatment resulted in a 10^3 decrease in the colony count of *S. aureus* and a 10^4 decrease in *E. coli* after 3 hr. Of important note was a decrease of more than 4 log-units in the *C. albicans* count after 24 hr.



Fig. 1. The Silano-Pen, commercially available as a dental bonding system.

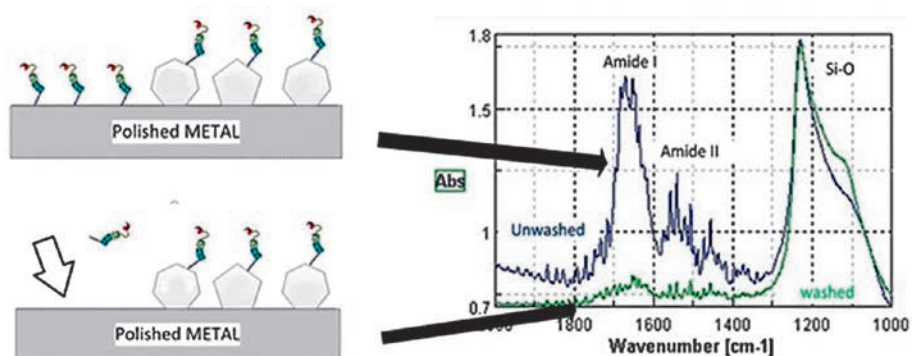


Fig. 2. Right panel shows the reflection-absorption spectroscopy Fourier-transform infrared (RAS-FTIR) spectrum of the titanium surface, before and after washing. Left panel shows the hypothesis: Protamine molecules are attached on metal and silica (upper). Water stream (white arrow) washes protamine off the metal surface. Even if protamine is attached to the silica, it does not appear in the RAS-FTIR spectrum (lower).

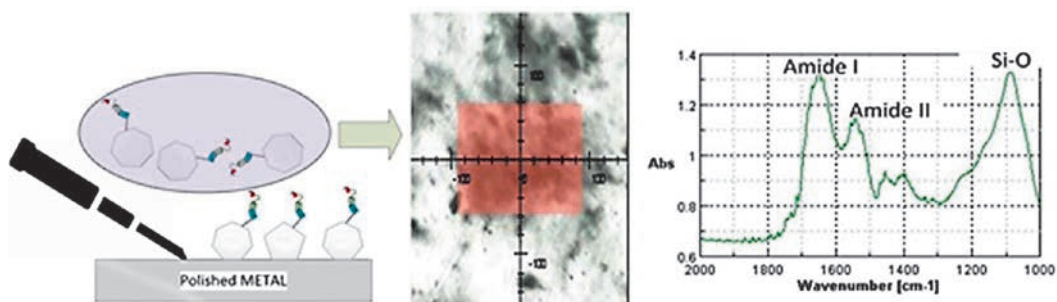


Fig. 3. Scraping of the surface of the plate with a chisel (left panel); microscopic view of the scraped and collected powder packed with potassium bromide powder (middle panel); and a micro Fourier-transform infrared spectrum showing amide peaks, which is different from the lower spectrum in Fig. 2, despite being the same (right panel).

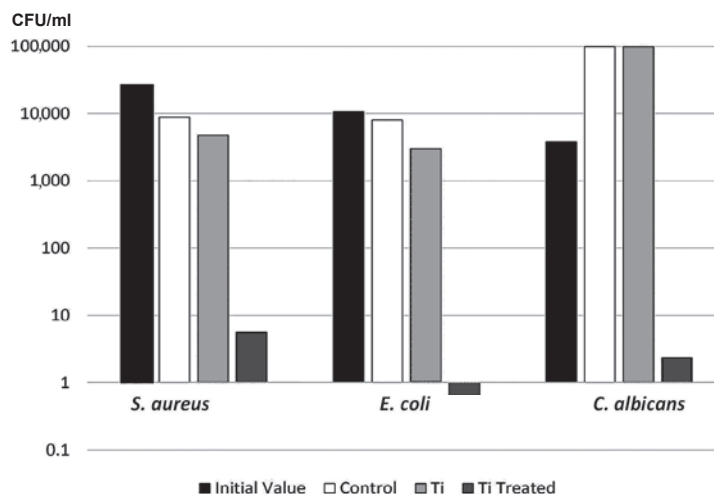


Fig. 4. Results of the JIS Z 2801 antimicrobial tests. Bacteria were cultured in contact with the surface of the material, and the number of live bacteria was counted. Ti, titanium.

Acrylic denture surface treatment

We investigated the treatment of dentures with the Silano-Pen to determine whether silica could be applied without damaging the denture (for subsequent application of antimicrobial protamine).

Figure 5 shows the flame of the Silano-Pen (upper, normal; lower, thermography). Thermography shows that the flow of silica particles rises sharply at the tip of the flame, even though the flow is invisible to the human eye. The flame cannot be brought close to the denture surface as it would cause burning. Therefore, the denture treatment should be carried out from just below the denture at a distance of 10 cm, so that the temperature of the denture surface does not exceed 100°C, and the denture will not be damaged (Fig. 6). In this case, the treated area was 6–7 cm in diameter.

Discussion

Many household goods, such as wallpaper, bath tubs, refrigerators, cutting boards etc., have passed the JIS Z 2801 test and are considered to have antimicrobial properties. The method reported in this paper achieved antimicrobial properties equal to, or better than, those described above.

However, the environment of the oral cavity is much harsher than the home living environment, and the antimicrobial coating needs to withstand any side effects and fungal change phenomena, while not affecting the intestinal and oral cavity bacterial flora.

A simple static electric force with ionic bonds causes binding between silica and protamine¹⁶⁾. Protamine is a cationic peptide; several antimicrobial materials are cationic. For example, hyssatin and cystatin are saliva peptides, and polylysine is a food preservative (as is protamine)²¹⁾. It is suspected that these substances might act in the same manner as protamine. However, more

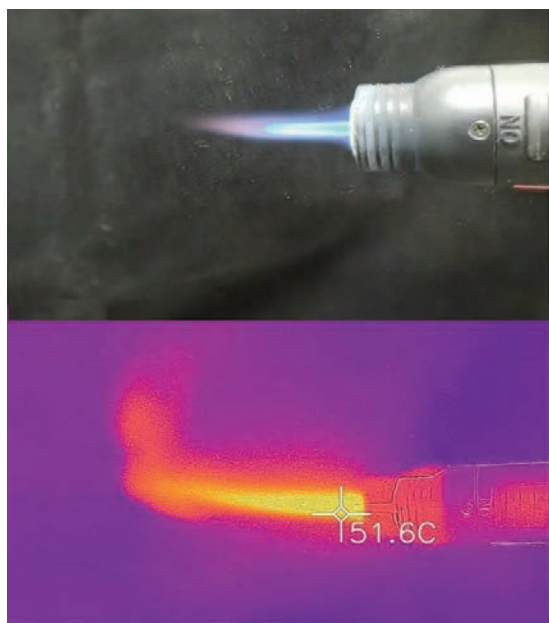


Fig. 5. Silano-Pen: normal view (upper); thermal image (lower). Silica vapor is invisible, but when taken with an infrared camera it can be seen to rise vertically from the tip of the flame.

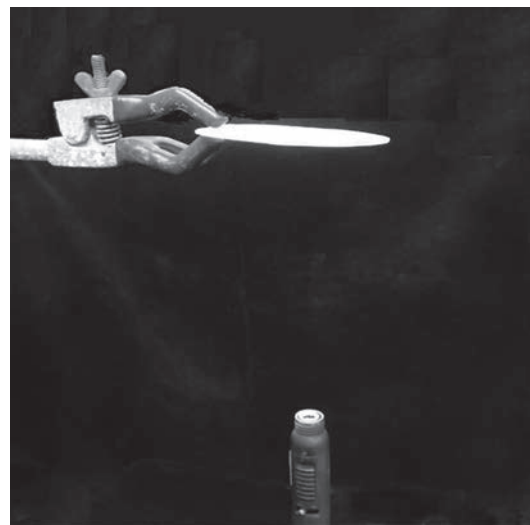


Fig. 6. The Silano-Pen should be used with the tip angled upwards, at a distance of 10 cm from the resin material.

research is needed to confirm this hypothesis.

The method described in this paper is limited in its efficacy; nevertheless, the materials are easy to apply (just blow and soak), removable (polish as usual or remove peptides with chemicals or enzymes), exchangeable (protamine from other species, other useful peptides), appendable (just rinse and soak) and safe (all materials used are food or food additives). Therefore, the authors of this study believe the findings described here could be useful in reducing the incidence of infectious diseases derived from artificial medical devices.

Conclusion

Protamine can be adsorbed onto silica-treated titanium surfaces. The number of live *S. aureus*, *E. coli* and *C. albicans* decreased by 1,000 fold (or more). Silica-bound protamine had a wide antimicrobial spectrum, including fungi. However, further investigation is needed to confirm its clinical efficacy.

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Conflicts of interest disclosure

The authors have no conflicts of interest to disclose.

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