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Antifungal Effects of a Tissue Conditioner Coating Agent with TiO₂ Photocatalyst

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Tissue conditioners are susceptible to colonization by microorganisms. Therefore, the prevention of biofilm formation are important for oral hygiene. However, mechanical and chemical cleaning methods may cause clinical problems such as deformation or surface degradation of tissue conditioners. The objective of this study is to evaluate the antifungal effects of coating agents with a TiO₂ photocatalyst. Photocatalytic antifungal effects on *C. albicans* biofilms and photodegradation effects of adsorbed protein were measured by colorimetric assays. Scanning electron microscopy was used to examine morphological changes in *C. albicans*. Viscosities of coating agents increased with incorporation of TiO₂. However, both of coating agents with TiO₂ were acceptable to the application by brush. The antifungal and protein degradation effects increased with the concentration of TiO₂ in the coating agents. These effects also increased with radiation time. After 90 min radiation, the viability of *C. albicans* was reduced to 16.2±3.3 %. Scanning electron microscopy observation showed *C. albicans* remained on the coated surfaces even after 90 min radiation. These results suggest that coating agents with TiO₂ photocatalyst can be effective for the maintenance of tissue conditioners when den-

tures are removed; during sleep.

Key words: tissue conditioner, coating agent, TiO₂ photocatalyst, antifungal effect, *Candida* biofilm

Introduction

Tissue conditioners that improve the health of abused denture-bearing tissues are more susceptible to colonization by microorganisms than denture base resins¹. Most microorganisms colonizing on the surface are found as complex-structured microbial communities (biofilms) and may irritate the underlying tissue²⁻⁴. Therefore, maintenance of tissue conditioners and the prevention of biofilm formation are important for oral hygiene. However, mechanical and chemical cleaning methods may cause clinical problems such as deformation or surface degradation of tissue conditioners^{5,6}.

Several studies have attempted to incorporate antibacterial agents into tissue conditioners for plaque control and treatment of denture stomatitis. However, the elution behavior of antibacterial agents has not been completely clarified and the salivary pellicles may inhibit the antibacterial agents from contacting with microbes⁷⁻¹⁰.

A TiO₂ photocatalyst, which produces free radicals on its surfaces by adequate light activation, has come to utilization such as self-cleaning¹¹, antibacterial¹², and waste water purification¹³ component. Additionally, a TiO₂ photocatalyst is chemically and physically stable and safe enough to be used as an additive for foods

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Received July 27; Accepted September 9, 2005

and cosmetics. These advantages have drawn much attention recently in the field of dental materials and some studies have been performed concerning the antifungal effect¹⁴⁻¹⁶. However, antifungal effects on biofilms that show much stronger resistance to antibacterial agents than the planktonic form¹⁷⁻²⁰ have not yet been reported in the previous studies.

We incorporated a TiO₂ photocatalyst into a coating agent for a tissue conditioner because it is important to expose a great number of TiO₂ particles on the surface of the tissue conditioner. In addition, coating agents are an effective method to reduce plaque accumulation of tissue conditioners^{21,22}. The purpose of this study was to investigate the antifungal effects of coating agents with a TiO₂ photocatalyst on biofilms. In the successful colonization and development of pathogenesis, the adherence of *C. albicans* to polymers such as soft lining materials is essential and necessary first step, followed by the growth of adherent cells or the co-adhesion of floating cells to adherent ones^{23,24}. Therefore we examined the viability of biofilm-grown *C. albicans* and observed *C. albicans* on the radiated coating surfaces by scanning electron microscopy (SEM). Moreover, we investigated the degradation of adsorbed protein that promotes microorganism accumulation²⁵.

Materials and Methods

Specimen preparation

The TiO₂ was anatase type (rutile type was less 50 %) and average particle size 10 nm (SHOWA DENKO, Tokyo, Japan). The TiO₂ powder was incorporated with polyethyl methacrylate based coating agents (TOP COAT; NISSIN, Kyoto, Japan). Three coating agents with different concentration of TiO₂ were prepared (Table 1). Tissue conditioner (FICTIONER; NISSIN, Kyoto, Japan) was processed according to the manufacturer's direction and prepared as disk-shape specimens (20 mm in diameter, 1 mm in thickness). Each specimens was painted with coating agents one

times on all surfaces and then stored in the dark for 15 min at room temperature.

Yeast strain and growth condition

C. albicans strain (JCM 1542; Physico-chemistry Institute, Saitama, Japan) was used as the biofilm forming yeast. Cells were grown for 24 h at 37 °C in a yeast nitrogen base medium (YNB medium; Difco Laboratories, Detroit, USA) supplemented with 50 mM glucose. Cells were harvested, washed with phosphate-buffered saline (PBS, pH 7.2), and adjusted to 1×10⁷ cells/mL.

Artificial candidal biofilm formation

Artificial candidal biofilms were formed on specimens according to Chandra J. et al.²⁰ with some modifications. Specimens were placed in each well of 12-well tissue culture plates and incubated for 2 h at 37 °C in 1 mL of 1 % bovine serum albumin (BSA; Sigma Aldrich Japan, Tokyo, Japan). After this pretreatment, 80 μL of *C. albicans* cell suspension was applied to the surfaces of specimens placed in 12-well tissue culture plates. Cells were allowed to adhere for 90 min at 37 °C (adhesion phase). Specimens were then immersed in 4 mL of YNB medium with 50 mM glucose and incubated for 48 h at 37 °C (biofilm growth phase).

Antifungal assay

After incubation, the YNB medium was removed and 4 mL of PBS was added to each well of the plates. The specimens with biofilms were radiated with an ultraviolet-A light source (365 nm, 1 mW/cm²) for 60 and 90 min. Subsequently, the plates were placed in an ultrasonic bath (45W, VC-1; AS ONE, Osaka, Japan) for 15 min to collect *C. albicans* cells from the specimens.

The viability of collected cells was determined by a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan), which is based on bioreduction of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) that produces a water-soluble formazan dye. The formazan dye decreases with reduced viability of biofilm-grown *C. albicans*. Ninety-six-well plates containing 50 μL of the collected cell suspension per well were incubated with 50 μL of the YNB medium with 50 mM glucose and 10 μL of CCK-8 prepackaged solution for 5 h at 37 °C. After incubation, absorbance at 450 nm was measured using a microplate reader (VersaMax; Molecular Devices Japan, Tokyo, Japan). The viability percentage was normalized to the 100 % conversion observed in nonradiated specimens.

Table 1. Component of coating agents
PEMA: polyethyl methacrylate

| | TiO ₂ (g) | PEMA (g) | ethyl acetate (g) |
|-----------------|----------------------|----------|-------------------|
| Coating agent 1 | 0 | 2 | 20 |
| Coating agent 2 | 2 | 2 | 20 |
| Coating agent 3 | 3 | 2 | 20 |

Scanning electron microscopy observation

Specimens were incubated for 24 h at 37 °C in suspension of *C. albicans*. After incubation, the specimens that were nonradiated or radiated with an ultraviolet-A light for 90 min were examined by SEM (JSM-5400; JEOL, Tokyo, Japan). Briefly, the specimens were placed in 2.5 % glutaraldehyde overnight, then rinsed in PBS and placed in 1 % osmium tetroxide for 3 h. Specimens were subsequently rinsed in distilled water, freeze-dried with liquid nitrogen and then coated with gold/palladium. After processing, specimens were observed using SEM.

Protein degradation assay

In previous artificial candidal biofilm formation process, BSA was used to substitute the salivary protein on *in vivo* biofilm formation. The effect of TiO₂ on BSA degradation was also evaluated.

One hundred μ L of 0.5 % BSA was applied to the surfaces of specimens placed in 12-well tissue culture plates. The specimens were radiated with an ultraviolet-A light source (365 nm, 1 mW/cm²) for 60 and 90 min. After radiation, the plates with 1 mL of 0.5% sodium dodecyl sulfate per well were placed in the ultrasonic bath for 15 min to collect residual BSA and decomposition products of BSA from the specimens.

The collected residual BSA and decomposition products were measured by a Protein Quantification Kit-Wide Range (PQK; Dojindo, Kumamoto, Japan), which is based on the reduction of tetrazolium salts. Absorbance has a correlation with a concentration of protein. The colorimetric assay was performed according to the manufacturer's direction and absorbance at 650 nm was measured using the microplate reader.

Statistical analysis

The effects of TiO₂ concentration and illumination time were analyzed using two-way ANOVA and Tukey's test at a 0.05 probability level. Each experiment was repeated five times. Two independent variables were TiO₂ concentration and illumination time. The dependent variables were the data of antifungal assay and protein degradation assay respectively.

Results

Viscosities of coating agents increased with incorporation of TiO₂. However, both of coating agents with TiO₂ could be applied to tissue conditioners by brush.

Incorporated TiO₂ particles were found on the coated surface (Figure 1).

Antifungal assay

Figure 2 shows relations between the viability of *C. albicans* in biofilms and radiation time. The viability significantly decreased with increasing the concentration of TiO₂ in the coating agents ($P < 0.05$). The viability also significantly decreased with radiation time ($P < 0.05$). After 60 min radiation, coating agent 2 showed no significantly antifungal effects in comparison with coating agent 1 (coating agent 1: 88.8 ± 4.5 %, coating agent 2: 88.6 ± 1.8 %). However, significant decreases

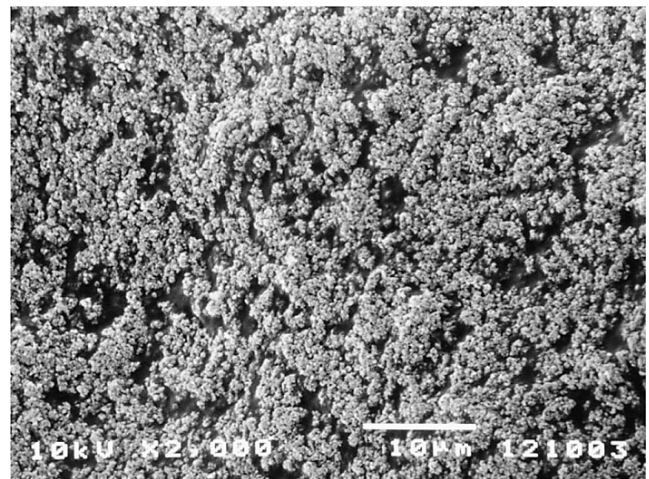


Fig. 1. SEM image of coating agent 3 ($\times 2000$) TiO₂ particles were observed on the surface of coated tissue conditioner.

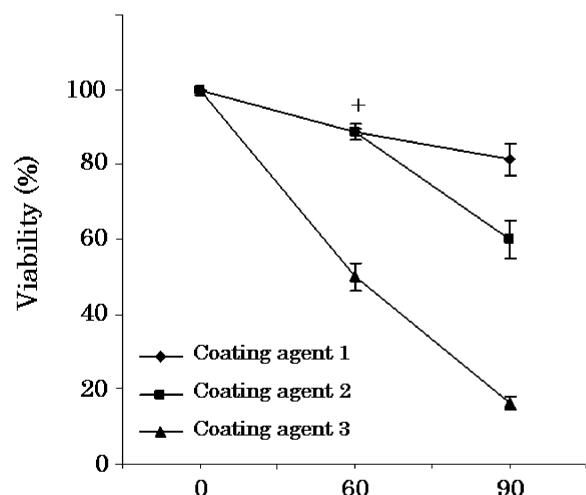


Fig. 2. Viability of *C. albicans* in antifungal assay Error bar indicates ± 1 SD. A single plus indicates no significantly difference between the coating agent 1 and 2 ($P > 0.05$).

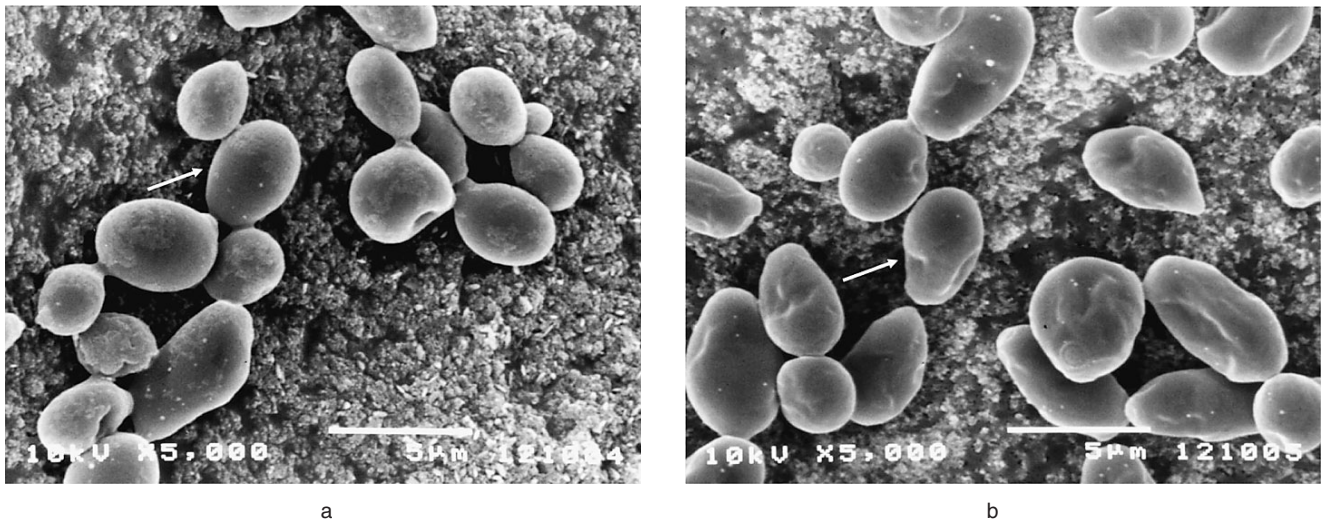


Fig. 3. SEM of *C. albicans* on coating agent 3 ($\times 5000$)
 a. Image of *C. albicans* on the coated surface before radiation (the arrow).
 b. Image of *C. albicans* on the coated surface after 90 min radiation (the arrow).

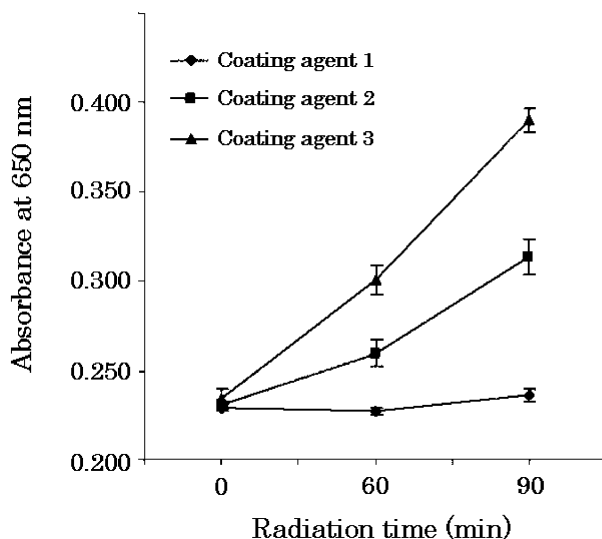


Fig. 4. Absorbance in protein degradation assay
 Error bar indicates ± 1 SD.

were observed after 90 min in radiated coating agent 2 (coating agent 1: 81.4 ± 8.5 %, coating agent 2: 60.1 ± 10.5 %, $P < 0.05$). Coating agent 3 showed a significant greater decrease of the viability of *C. albicans* than other coating agents ($P < 0.05$). After 90 min radiation, the viability of *C. albicans* was reduced to 16.2 ± 3.3 %.

Scanning electron microscopy observation

Figure 3 shows SEM analysis of *C. albicans* on non-radiated and radiated coated surfaces of coating

agent 3, which showed most marked antifungal effect in this study. After 90 min radiation, fungous bodies of *C. albicans* remained on the coated surfaces of tissue conditioners. However, many *C. albicans* on the radiated surfaces had dents and irregular contours.

Protein degradation assay

Figure 4 shows the absorbance in protein degradation assay. The absorbance significantly increased with the concentration of TiO_2 in the coating agents ($P < 0.05$). The absorbance also significantly increased with radiation time ($P < 0.05$). The absorbance of coating agent 3 showed a significantly greater increase than that of coating agent 2 ($P < 0.05$).

Discussion

Coating agents with TiO_2 showed significant decreases in the viability of *C. albicans*. The photocatalytic antifungal effects result from denaturation of *Candida* cell walls because many yeast cells on the radiated surfaces had dents in SEM observation. SEM examination observed that fungous bodies of *C. albicans* remained on the radiated surfaces, suggesting that rinsing out the radiated surfaces is important to prevent more microorganisms accumulating.

A TiO_2 photocatalyst activated by UV can only decompose protein in contact with the TiO_2 , and therefore, decomposition products were derived from adsorbed BSA. The decomposition products are

smaller molecular size protein than BSA because a TiO₂ photocatalyst acts on the amide bond of protein¹³. The absorbance of radiated conditions with TiO₂ significantly increased with radiation time. The results showed that the coating agents with greater amounts of TiO₂ had greater degradation effects on the adsorbed protein.

In this study, coating agent 2 that showed significant degradation of BSA indicated no antifungal effects after 60 min radiation. This might have been caused that the radicals from photocatalytic reactions were expended decomposing the extracellular matrix of the biofilm. However, the elimination of the biofilms on the coated surfaces with TiO₂ may be easy because it elevates the hydrophilicity after radiation and decomposes adsorbed protein that promote microorganism accumulation. It is of interest to establish whether the coating agents with TiO₂ are effective the maintenance of tissue conditioners by short radiation time and studies are recommended.

Antibacterial agents incorporated into coating agents in the previous studies consistently act on oral flora. However, TiO₂ shows no photocatalytic effects at nonradiation. So that means the coating agents with TiO₂ may have no antifungal effect in oral cavity environment. When dentures are removed, for instance during sleep, the photocatalytic coating agents allow for the maintenance of tissue conditioners by just radiating the coated surfaces. This will be useful in an aging society that requires simple and easy cleaning methods.

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