

Evaluation of antibacterial activity of visible light-responsive TiO₂-based photocatalyst coating on orthodontic materials against cariogenic bacteria

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Purpose: The objectives of this study were to evaluate antibacterial activity of visible light-responsive photocatalytic titanium dioxide (Vis-TiO₂) to cariogenic bacteria.

Materials and Methods: In all analyses, brookite Vis-TiO₂ was coated on stainless steel surfaces, which were illuminated with visible light (405 nm; LED-generated). Antibacterial activity against cariogenic bacteria was assessed by colony-forming unit (CFU) assay, radial diffusion assay, and bacterial viability analysis.

Results: CFU assay demonstrated that the number of colonies of both bacteria on coated plates with illumination reduced to around 10 %. The coated materials produced clear zones in culture petri dishes of both cariogenic bacterial species. Fluorescence microscopy revealed that most of the bacteria on the coated plates with illumination was identified as dead. In flow cytometry analysis, more than 68% of *S. mutans* and more than 91% of *S. sobrinus* were killed on the coated plates with illumination. Vis-TiO₂ has strong bactericidal effects on both cariogenic bacteria after illumination. Water-insoluble glucan on the coated plate with illumination was significantly less formed.

Conclusion: The visible light-responsive photocatalytic titanium dioxide coating on orthodontic materials has strong antibacterial activity, which is based primarily on bactericidal activity. The Vis-TiO₂ coating is useful for dental caries prevention without any adverse effect to the human body.

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Key Words: antibacterial, bactericidal activity, dental caries, orthodontic material photocatalysis, visible light

Introduction

Dental caries is one of the most prevalent diseases in the world [1], and also one of the serious side effects during orthodontic treatment [2]. The caries around the orthodontic brackets, which are developed by enamel demineralization, have been a critical problem in daily orthodontic treatment [3]. It was caused by prolonged accumulation of bacterial biofilm on the enamel surfaces around the orthodontic brackets. Most of the biofilm is initially formed on the surface of orthodontic bracket and spread on the enamel surface around the bracket. Therefore, it is clinically important to inhibit the formation of biofilm on the orthodontic bracket [4].

The biofilm is formed by cariogenic bacteria, *Streptococcus mutans* and *Streptococcus sobrinus* [5-10]. Therefore, it has been clinically important to inhibit the activity of cariogenic bacteria. However, it is difficult for the orthodontic patients to clean the brackets with complex forms on their teeth during orthodontic treatment; such cleaning requires a great deal of compliance by the patients.

There have been several studies on the prevention of dental caries. One potential strategy is to coat the orthodontic brackets with antibacterial reagents such as photocatalytic TiO₂ and to irradiate them with ultraviolet light (UV) [11,12]. The release of reactive oxygen species (ROS) from the photocatalytic TiO₂ inhibits the growth of cariogenic bacteria [13]. However, the use of UV irradiation is dangerous, since UV is harmful to oral mucosa and skin [14].

Recently, LED light has been shown to have the potential to activate photocatalytic TiO₂ [15-17]. Therefore, the purpose of this study was to evaluate the antibacterial activity of visible light-responsive photocatalytic TiO₂ against cariogenic bacteria with the activation by LED visible light which has no adverse effect to the human body.

Materials and Methods

Sample preparation

The materials used in this study consisted of stainless steel disks (5.0 mm diameter x 3.0 mm thick) and square plates (100.0 x 100.0 mm). The surface roughness (Ra) were measured using a confocal laser scanning microscope (OLS 3000, Olympus Co., Tokyo, Japan). Ra of the stainless steel was Ra 2.14 ± 0.097 μm, which is the same as that of orthodontic brackets (Metal bracket, Densply Sankin, Tokyo, Japan: Ra 2.12 ± 0.174 μm).

A dip-coating method (using 3 wt% TiO₂ brookite-rich sol; NTB-13, Showa Denko, Tokyo, Japan) was used to apply visible light-responsive photocatalytic titanium dioxide (Vis-TiO₂) to the surfaces of the materials [18]. The coated materials were heated at 100°C for 15 min to modify the TiO₂ nanoparticles onto the surface. After heating, the excess coating agent was removed in the ultrasonic device. Then during drying the temperature was maintained under 100°C in order to prevent the inactivation of brookite TiO₂. Ra after coating was 2.00 ± 0.154 μm, indicating that there was no statistical difference before and after coating. All materials were sterilized with ethylene oxide gas before use.

The illumination device used in this study consisted of an array of 90 small LED illuminator bulbs (Fig. 1), and emitted visible light at 405 nm (SPL-90-CC, Revox, Sagamihara, Japan). The distance between LED and bacteria in all of this study was 35 mm, and the intensity of the light was 180 Lx.

Bacterial strains

Cariogenic bacteria *Streptococcus mutans* ATCC 25175 (*S. mutans*) and *Streptococcus sobrinus* ATCC 33478 (*S. sobrinus*) were used in this study. Both bacteria were inoculated into 4 mL of Tryptic Soy (TS) broth (Becton, Dickinson and Company, Sparks, MD, USA) and were cultured at 37°C for 16 h.

Colony-forming unit (CFU) assay

Bacterial cells from the culture medium were washed with PBS and the aliquots (1.5 mL) of the each bacterial suspension were poured on the surface of the coated and non-coated square plates on ice, and light illumination was performed for 2 h. After illumination, bacterial cells were harvested by centrifugation (1,750×g, 4°C, 20 min). Each bacterial cell pellet was resuspended in 1 mL PBS and subjected to serial 10-fold dilutions in PBS. The dilutions of each bacteria were inoculated on MS agar (Difco Mitis Salivarius Agar (semi-selective medium for streptococci); BD Biosciences, Franklin Lakes, NJ, USA) in petri dishes with spiral plating equipment (Eddy Jet, IUL SA, Barcelona, Spain), and petri dishes were incubated under anaerobic conditions in an AnaeroPack-Anaero box (AnaeroPack System, Mitsubishi Gas Chemical Co. Inc, Tokyo, Japan) at 37°C for 48 h. The number of colonies was calculated in accordance with the spiral plater instruction manual.

Radial diffusion assay

Radial diffusion assays [19] were performed as follows. The coated and non-coated disks (5.0 mm) mentioned above were placed on petri dishes. TS agar medium (6 mg TS and 100 mg agarose in 10 mL distilled water) was sterilized by autoclaving and cooled to 50°C. Bacterial cells collected by centrifugation from TS culture medium were added in sterilized TS agar medium at a cell density of OD₅₄₀ = 1.0 (corresponding to 100 μL or 300 μL of

S. mutans or *S. sobrinus* culture, respectively). The TS agar medium containing bacterial cells was poured over the disks in the dishes (achieving a depth sufficient to submerge the disks), and then another aliquot of enriched TS agar medium (600 mg TS and 100 mg agarose in 10 mL distilled water) was added on the pre-poured TS agar medium.

After light illumination for 2 h in the air at room temperature, the bacteria in the dishes were incubated in the dark under anaerobic condition at 37°C for 24 h using an AnaeroPack-Anaero box. After incubation, diameters of the clear zones of bacterial inhibition around the disk materials were measured with a slide calipers. Antibacterial activity was calculated as follows; Antibacterial activity (mm) = diameter of clear zone (mm) – diameter of disk (mm) [20].

Bacterial viability analysis by fluorescent microscopy and flow cytometry

The viability of bacterial cells on each square plate was evaluated by the fluorescence microscopy (Olympus AX 80; Olympus Co. Ltd., Tokyo, Japan) using the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Carlsbad, CA, USA). Briefly, bacterial cells (*S. mutans* or *S. sobrinus*) from the culture medium were washed with PBS; 50 µL of the suspension of either species were combined with 0.5 µL of the BacLight reagent. This staining solution contains SYTO 9, a cell membrane-permeable green fluorophore, and PI, a cell membrane-impermeable red fluorophore. In this fluorescence system, viable bacterial cells exhibit green fluorescence, while nonviable cells exhibit red fluorescence. The excitation/emission maxima for these dyes are about 480/500 nm for SYTO 9 stain and 490/635 nm for PI. Bacterial cells were observed by fluorescence microscopy using a 470/490 nm excitation cube.

Furthermore, the bacterial cells were quantified by flow cytometry using a BD Accuri C6 flow cytometer (BD, Bioscience, Franklin Lakes, NJ, USA) [21,22]. The stained bacterial cells were excited using a 488-nm solid-state laser, and fluorescence was detected using 533/30 BP (FL-1 channel) and 670 LP filters (FL-3 channel) for SYTO 9 and PI, respectively. The bacterial fraction (SYTO 9-positive population) was gated on an SSC/FL1 plot, and fluorescence of PI was monitored in the FL-3 channel.

Effects of Vis-TiO₂ coating on cariogenic biofilms

The inhibitory effects of the TiO₂ coating on cariogenic biofilm formation were examined on the surfaces of the disks using separate suspensions of *S. mutans* and *S. sobrinus*. The culture medium for biofilm formation was prepared using TS broth without dextrose, which was supplemented with 0.5% sucrose (Wako, Osaka, Japan) (TSS broth). The pre-cultured suspensions of *S. mutans* or *S. sobrinus* were added to TSS broth. Then the sterile disks ($n = 5$ for each bacterial species) were incubated in 500 µL of the above medium in a 48-well culture plate under continuous LED light illumination for 24 h at 37°C.

The disks were retrieved from the medium, gently washed three times with sterile phosphate-buffered saline (PBS; pH 7.4), soaked into 1 mL of 0.5 N NaOH in a glass test tube (Iwaki, Tokyo, Japan) for 10 min to solubilize water-insoluble glucan (WIG), and the WIG was completely detached from the disks by vortexing. After picking out the disks, the suspensions in the tubes were centrifuged (1,750×g, 4°C, 15 min) to pellet of the bacteria. WIG content in the supernatants was analyzed using the phenol-sulfuric acid method [23,24].

Statistical analysis

Statistical analyses were performed using SPSS 22.0 for Windows (SPSS Inc., Version III, Chicago, IL, USA). Amount of biofilm were compared using Kruskal-Wallis and Mann-Whitney *U*-tests. The results of radial

diffusion assays, and CFU assays were compared using the Student's *t*-test. Differences were considered to be significant at $p < 0.05$.

Results

Antibacterial activity by UV-responsive TiO₂ has been shown in many studies [3,25-27]. However, there have been few studies on the antibacterial activity by Vis-TiO₂. In this study we examined whether Vis-TiO₂ coated on the orthodontic materials has antibacterial activity against cariogenic bacteria.

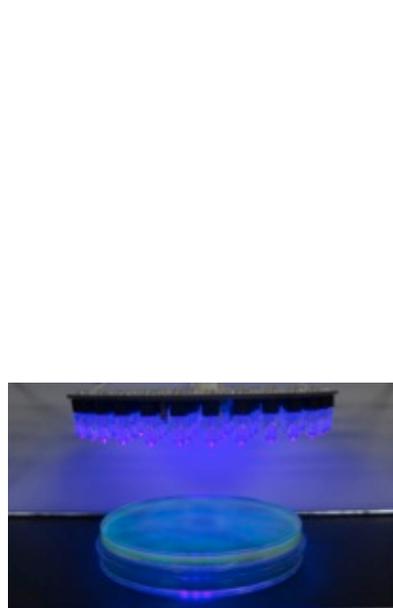


Fig. 1
LED illuminator-bulbs for visible light

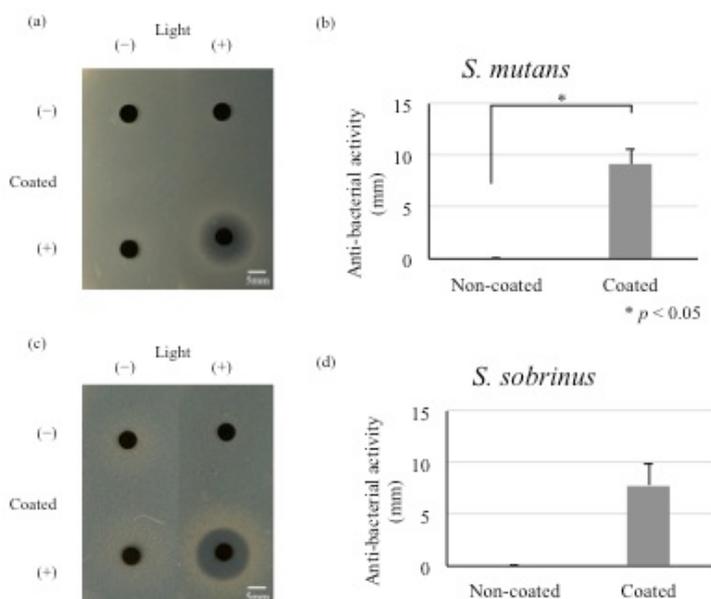


Fig. 2
Anti-bacterial activity of coated and non-coated disks ($n = 5$) against *S. mutans* (a, b) and *S. sobrinus* (c, d) with and without illumination by radial diffusion assay. The asterisks indicate statistically significant differences ($p < 0.05$).

We examined the effect of Vis-TiO₂ on the formation of colony by cariogenic bacteria. CFU assay showed that the antibacterial activity on the coated materials with visible-light illumination induced a significant reduction in the number of colony. The number of *S. mutans* was reduced from 448.8 to 7.3 and that of *S. sobrinus* from 417.5 to 8.5, and consequently about 95% of colony by both *S. mutans* and *S. sobrinus* was not formed on the coated plates with illumination (Table 1).

Table 1 The CFU of *S. mutans* and *S. sobrinus* on coated and non-coated plate materials after illumination for 2 h

	<i>S. mutans</i>	<i>S. sobrinus</i>		<i>S. mutans</i>	<i>S. sobrinus</i>
Non-coated, Light (-)	440.2 ± 12.9	394.7 ± 23.9	Coated, Light (-)	450.4 ± 39.0	420.7 ± 33.5
Non-coated, Light (+)	448.8 ± 27.9	417.5 ± 28.9	Coated, Light (+)	*7.3 ± 1.0	*8.5 ± 0.7

The asterisks indicate statistically significant differences ($p < 0.01$) compared to non-coated condition in each strain ($n = 5$).

Antibacterial activity of the Vis-TiO₂ was also confirmed by radial diffusion assay [28,29]. This assay determined the area influenced by the Vis-TiO₂. The results demonstrated that no detectable clear zone was observed around the coated and non-coated materials without illumination or around non-coated material with illumination in the *S. mutans* or *S. sobrinus* cultured petri dishes (Figs. 2a and 2c). However, the coated materials with illumination evidently produced clear zones in both *S. mutans* and *S. sobrinus* culture petri dishes.

We further confirmed the antibacterial activity of the Vis-TiO₂ by detecting live and dead cells in the cultured bacterial cells. Fluorescence microscopy revealed that most of bacteria of both species on the non-coated plates with or without illumination was identified as alive as shown by green fluorescence (Figs. 3a-c and e-g). In addition, bacterial cells on coated plates without illumination was also alive. On the other hand, the bacteria on the coated plates with illumination was identified as dead, as shown by red fluorescence (Figs. 3d and 3h). These results indicate that the Vis-TiO₂ with illumination has strong bactericidal effects on both species of cariogenic bacteria. These results evidently indicate that most of the antibacterial activity of the Vis-TiO₂ is based on bactericidal activity [30], not the inhibition of bacterial growth.

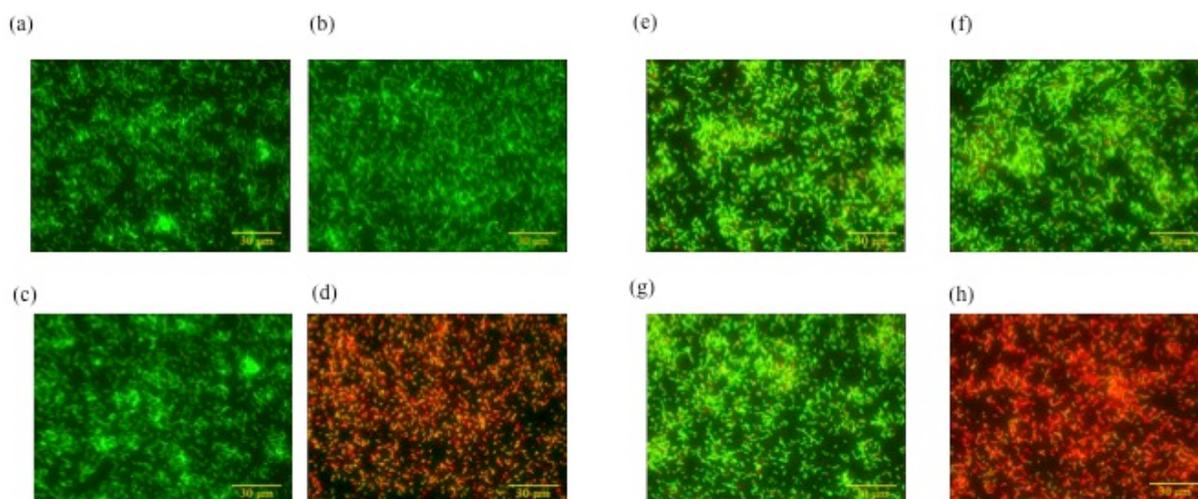


Fig. 3 Fluorescence staining of the populations of *S. mutans* (a, b, c, d) and *S. sobrinus* (e, f, g, h) on coated and non-coated plate materials ($n = 5$) after illumination for 2 h. (a, e): non-coated, light (-), (b, f): non-coated, light (+), (c, g): coated, light (-), (d, h): coated, light (+). Original magnification 400 \times , scale bar 30 μ m

Flow cytometry analysis quantitatively confirmed these results of *S. mutans* (Fig. 4a) and *S. sobrinus* (Fig. 4b). In each figure bacterial cells on non-coated plates showed fluorescence peak of FL-3 around 10^5 . Similar to them, bacterial cells on coated plates without illumination showed fluorescence peak of FL-3 around 10^6 . On the other hand, bacterial cells on coated plates with illumination showed the increase of fluorescence in FL-3, which indicates the increase of dead cells in the samples. The number of FL-3 negative cells was more than 96%, signifying bacterial cells non-coated on plates remained alive even after 2 h illumination (Table 2). In addition, the bacterial cells on the coated plate without illumination was also alive. However, more than 68% of *S. mutans* and more than 91% of *S. sobrinus* bacterial cells were FL-3 positive, *i.e.*, killed on the coated plate after 2 h of illumination.

Table 2 Percentages of live and dead bacterial cells of *S. mutans* and *S. sobrinus* on coated and non-coated plates with illumination in flow cytometry analysis

	<i>S. mutans</i>		<i>S. sobrinus</i>	
	Live (%)	Dead (%)	Live (%)	Dead (%)
Non-coated, Light (-)	97.27 \pm 0.0165	2.73 \pm 0.0165	98.28 \pm 0.0178	1.72 \pm 0.0178
Non-coated, Light (+)	96.26 \pm 0.0097	3.74 \pm 0.0097	98.58 \pm 0.0034	1.42 \pm 0.0034
Coated, Light (-)	96.66 \pm 0.0056	3.34 \pm 0.0056	98.57 \pm 0.0028	1.43 \pm 0.0028
Coated, Light (+)	*31.84 \pm 0.2850	*68.16 \pm 0.2850	*8.52 \pm 0.0335	*91.48 \pm 0.0335

The asterisks indicate statistically significant differences ($p < 0.05$) compared to non-coated, light (+) conditions in each strain of *S. mutans* or *S. sobrinus* ($n = 5$).

On the basis of these results, we examined whether the Vis-TiO₂ has an inhibitory effect on biofilm formation through antibacterial or bactericidal activity (Fig. 5). The amount of WIG on the coated disk with illumination was significantly less than those on the other three disks: non-coated without illumination, non-coated with illumination, and coated without illumination.

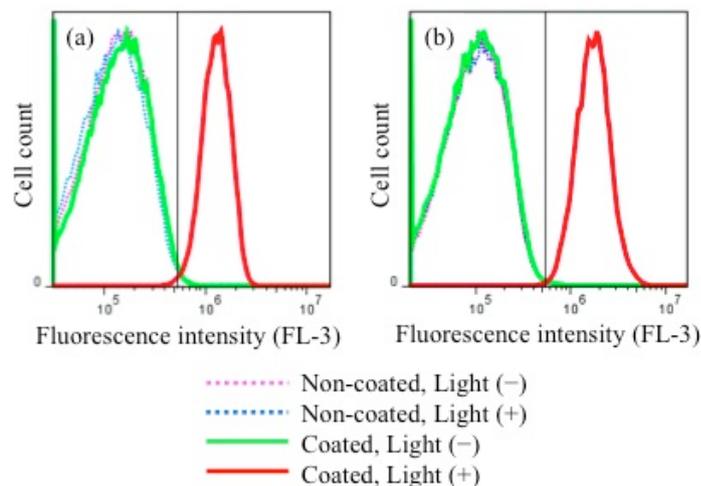


Fig. 4

Flow cytometry analysis of cell populations of *S. mutans* (a) and *S. sobrinus* (b) after illumination for 2 h ($n = 5$). X-axis indicates fluorescence intensity of FL-3 channel, and Y-axis indicates the number of cell count at each fluorescence intensity. Pink dot curve: non-coated, light (-), blue dot curve: non-coated, light (+), green curve: coated, light (-), red curve: coated, light (+). Vertical black line indicates threshold.

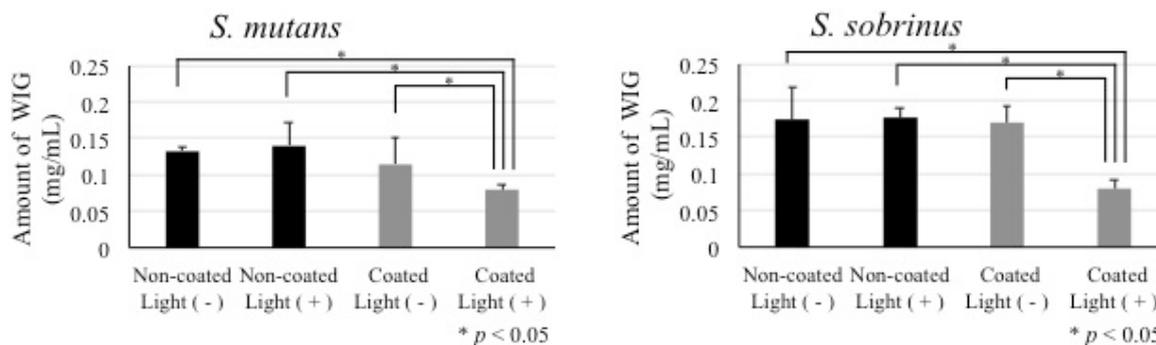


Fig. 5 Amount of WIG in the biofilm formed on coated and non-coated disks. *S. mutans* and *S. sobrinus* with or without illumination ($n = 5$). The asterisks indicate statistically significant differences ($p < 0.05$).

Discussion

S. mutans and *S. sobrinus* play a pivotal role in dental caries and they are inherently difficult to eradicate. Mechanical tooth cleaning by brushing is the main method for prevention of dental caries in contemporary orthodontic treatments. This has been performed for a long time, the consensus view is that patients generally are unable to brush well enough to remove plaque around multi-bracket appliances. There have been some studies on the antibacterial effects of orthodontic adhesive resins [31]. But the surplus of the adhesive resins during the bracket bonding is usually removed from the enamel surface, and consequently the antibacterial effects of the adhesive resins are not expected around the brackets.

Significant reduction of colony was observed in CFU assay (Table 1). The antibacterial activities of Visible-light responsive nickel-doped TiO₂ was investigated in the previous study [32]. In our study, we examined antibacterial activity of pure brookite-rich TiO₂, which showed similar high antibacterial activity. These results suggest that the Vis-TiO₂ in this study has notable ability to inhibit the growth of cariogenic bacteria.

The obtained from radial diffusion assay results were very interesting from the point of the antibacterial activity (Fig. 2). The activity of the Vis-TiO₂ was observed on the surface area of the coated disk and in the area some distance away from the disks. Thus, the Vis-TiO₂ has potential antibacterial activity against cariogenic bacteria not only on the coated surface but also in the area some distance away from the coated surface, which is one of the characteristic features of this study. This is probably due to the release of ROS from the coated disks, which diffuse around the disks with concentration gradient [33], and consequently inhibit the bacterial growth in the area away from the surface.

Fluorescent microscopy demonstrated that, the effect of Vis-TiO₂ was bactericidal activity (Figs. 3 and 4). The bactericidal activity is based on the fragmentation of organic materials by the photocatalyst. The TiO₂ on the plates generates ROS such as hydroxyl radical (\bullet OH), superoxide radical ($O_2^{\bullet-}$) [34], and singlet oxygen (1O_2) [35], under visible LED light illumination. The bactericidal activity of the Vis-TiO₂ could be caused by the strong oxidative activity of ROS.

WIG is the essential component of biofilm, which generates strong adherence property to the surfaces of the materials and is usually produced by the cariogenic bacteria by polymerization of glucose derived from sucrose in the food [36-40]. The Vis-TiO₂ has the strong ability to reduce biofilm formation on the coated disk with illumination (Fig. 5). These results indicate that the Vis-TiO₂ inhibits the formation of biofilm, which result from antibacterial activity to cariogenic bacteria. This is expected to be advantageous for the prevention of caries around orthodontic brackets. Vis-TiO₂ has potential application for the prevention of dental caries through the inhibition of biofilm formation not only in orthodontics, but also in dentistry.

The visible light-responsive photocatalytic titanium dioxide coating on orthodontic materials has strong antibacterial activity, which is based primarily on bactericidal activity. The visible light-responsive photocatalytic titanium dioxide coating is useful for dental caries prevention without any adverse effect to the human body.

Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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