Original Article

The effect of irradiation wavelengths and the crystal structures of titanium dioxide on the formation of singlet oxygen for bacterial killing

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Safe and effective methods for oral bacterial disinfection have been desired, since bacteria cause many infectious diseases such as dental caries, periodontal disease, and endodontic infections. Singlet oxygen ($^1O_2$) is attractive, because it is toxic to prokaryotic cells, but not to eukaryotic cells. We selected irradiation of titanium dioxide (TiO_2) as a source of $^1O_2$, because it has been used in sunscreens and cosmetic products without complications. In order to establish the optimal oral photodynamic therapy conditions, we measured the rate of $^1O_2$ formation from the irradiated anatase or rutile forms of TiO_2 using 365 or 405 nm lamps. The rate of $^1O_2$ formation decreased in the following order: anatase, 365 nm > rutile, 405 nm > rutile, 365 nm > anatase, 405 nm. Therefore, we concluded that irradiation of the rutile form of TiO_2 by a 405 nm lamp is the most favorable photodynamic therapy condition, because visible light is more desirable than UV light from the viewpoint of patient safety. We also confirmed that there was no direct $HO^•$ formation from the irradiated TiO_2.

Key Words: **singlet oxygen, titanium dioxide, 405 nm, electron spin resonance spectroscopy, photodynamic therapy**

Oral bacteria cause many infectious diseases such as dental caries, periodontal disease, and endodontic infections.[1,2] It has also been demonstrated that oral bacteria are involved in various systemic diseases such as endocardial inflammation, aspiration pneumonitis, and diabetes,[3–5] and controlling these bacteria is a major challenge in dental therapy. Conventional chemical disinfection causes problems such as tissue damage and accidental injury due to the leakage of chemicals.[6,7] A material that causes no damage to the body and possesses selective bactericidal properties is highly desired.

Exogenous singlet oxygen ($^1O_2$) is toxic to prokaryotic cells, but is almost nontoxic to eukaryotic cells.[8,9] Nakano *et al.*[8] reported that exogenous $^1O_2$ induces damage to the electron transport systems located on the surface membrane of prokaryotic cells, but in eukaryotic cells, the electron transport systems is stored in the inner mitochondria membrane, which is inaccessible to the short-lived $^1O_2$.[9]

Recently, oral photodynamic therapy (PDT) utilizing $^1O_2$ has gained significant attention. $^1O_2$ is generated by irradiating photosensitizers such as methylene blue. Although this form of PDT is effective to eliminate oral bacteria,[2,10,11] methylene blue has been reported to be carcinogenic.[12] On the other hand, titanium dioxide (TiO_2) also produces $^1O_2$ following irradiation,[13–17] but it has been used in sunscreen for humans without complications.[13,15] Therefore, we evaluated the potential of using TiO_2 as a new photosensitizer for oral PDT.

TiO_2 has two crystal structures, the anatase and rutile. In order to establish the optimal PDT condition, we measured the rate of $^1O_2$ formation from irradiated anatase or rutile forms. We also compared the difference resulting from using the irradiation wavelengths between 365 and 405 nm.

Materials and Methods

Materials and light source. TiO_2 (anatase and rutile, particle size of 5 μm), 2,2,6,6-tetramethyl-4-piperidone (4-oxo-TMP) hydrochloride, and 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene, BHT) were purchased from Wako Pure Chem. Ind. Ltd. (Osaka, Japan). 5-(2,2-Dimethyl-1,3-propoxycyclophosphoryl)-5-methyl-1-pyroline-*N*-oxide (CYPMPO) was purchased from Radical Research (Tokyo, Japan). Superoxide dismutase (SOD) and 2,2,6,6-tetramethy-4-piperidone-N-oxyl (4-oxo-TEMPO) were purchased from Sigma Aldrich (St. Louis, MO). All other reagents were of analytical grade. The two LED light sources, λmax = 365 nm (2.18 mw/cm^2) and 405 nm (8.69 mw/cm^2), were obtained from J. Morita Mfg. Corp. (Kyoto, Japan).

ESR measurement. A suspension of TiO_2 (0.4–4 mg/ml) in phosphate buffer was mixed with 4-oxo-TMP or CYPMPO; the concentrations of 4-oxo-TMP and CYPMPO were 40 and 100 mM, respectively. A solution of SOD (10 U/ml) was added if necessary. All solutions were freshly prepared, mixed, and immediately transferred into a flat quartz ESR cell, and measured using an ESR spectrometer (JES JFA-200, JEOL, Tokyo, Japan). The ESR measurements were conducted under the following conditions: magnetic field, 330 ± 5 mT; modulation width, 0.05 mT; time constant, 0.03 s; microwave frequency, 9.420 GHz; microwave power, 4 mW; sweep width, 5 mT; sweep time, 1 min; and amplitude, 500. Signal intensities were normalized to a MnO marker, and the concentrations of stable radical products were determined by using an external standard based on the signal height.[18]

Photooxidation of uric acid and BHT. The photooxidation of 100 μM uric acid or BHT was conducted in the presence of TiO_2 (4.0 mg/ml) at room temperature under aerobic conditions.[13,15] Uric acid was quantified by HPLC separation on an aminopropylsilyl column (5 μm, 4.6 × 250 mm, Supelco, Sigma-Aldrich, Tokyo, Japan) using methanol/40 mM aqueous monobasic

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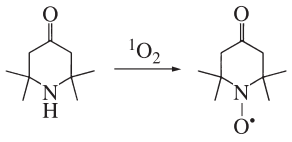
sodium phosphate (90/10) as the mobile phase (1 ml/min) with detection at 291 nm. BHT was measured similarly by UV detection at 277 nm after separation on an octadecylsilyl column (5 μm, 4.6 × 250 mm, Supelco, Sigma-Aldrich, Tokyo, Japan) using methanol/water (95/5) as the mobile phase (1 ml/min).

Results and Discussion

In order to establish the optimal PDT condition, we measured the rate of $^1O_2$ formation from the irradiated anatase or rutile forms of TiO_2 using 365 or 405 nm LED lamps. The production of $^1O_2$ was evaluated by the $^1O_2$ specific oxidation of 4-oxo-TMP to 4-oxo-TEMPO, which is detectable by ESR (eq. 1). Fig. 1 illustrates the time-dependent increase in ESR spectra obtained upon the irradiation of TiO_2 with 4-oxo-TMP. The ESR signal displayed a 1:1:1 triplet signal characteristic of 4-oxo-TEMPO having a hyperfine splitting constant (hfsc, aN = 1.608 mT).[16] Upon irradiation of the anatase form of TiO_2, the signal intensity was stronger when the 365 nm lamp was used instead of the 405 nm lamp (Fig. 1). However, the order was the opposite for the irradiation of the rutile form of TiO_2 (Fig. 1). These results were reproducible, as shown in Fig. 2.



(1)

Since uric acid is reactive to $^1O_2$, we compared the rate of uric acid consumption following the irradiation of TiO_2 (anatase or rutile) with the 365 or 405 nm sources (Fig. 3). The rate of uric acid consumption decreased in the order of the following combination: anatase, 365 nm > rutile, 405 nm > rutile, 365 nm > anatase, 405 nm. This order was identical to the order observed in the $^1O_2$ specific oxidation of 4-oxo-TMP (Fig. 2). It is noteworthy that the band gap energies of the anatase and rutile forms of TiO_2 are 3.2 and 3.0 eV, and therefore, photo-excitation should occur at wavelengths below 387 and 413 nm, respectively.[19,20] These values are consistent with the rate of $^1O_2$ formation observed in this study.

Based on the above results, we concluded that the irradiation of the rutile form of TiO_2 using a 405 nm lamp is the most favorable PDT condition, because visible light is more desirable than UV light from the viewpoint of patient safety. We are planning to apply these PDT conditions for oral bacterial disinfection.

To establish a safe method for PDT utilizing $^1O_2$, we need to confirm that there were no formation of hydroxyl radical ($HO^•$) following the irradiation. We used CYPMPO as an ESR spin-trapping probe, since it can distinguish between the formation of $HO^•$ and superoxide ($O_2^{•-}$).[21–23] The observed ESR spectrum following the irradiation of TiO_2 showed CYPMPO-OH adducts in all groups (Fig. 4).[21,24] However, this signal disappeared when SOD was added to the reaction. This implies that the CYPMPO-OH adduct was derived from $O_2^{•-}$, and $OH^•$ was not generated directly from the irradiated TiO_2.

To obtain more direct evidence of confirming that there was no formation of $HO^•$ in this system, we photooxidized BHT in an aqueous methanol solution in the presence of TiO_2.[13,15] If $HO^•$ is

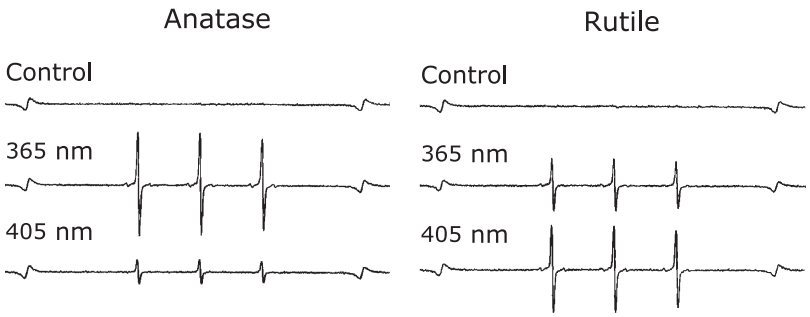


Fig. 1. The EPR spectra for the production of 4-oxo-TEMPO following irradiation of the $^1O_2$-specific reagent, 4-oxo-TMP (40 mM) with TiO_2 (4.0 mg/ml) for 5 min.

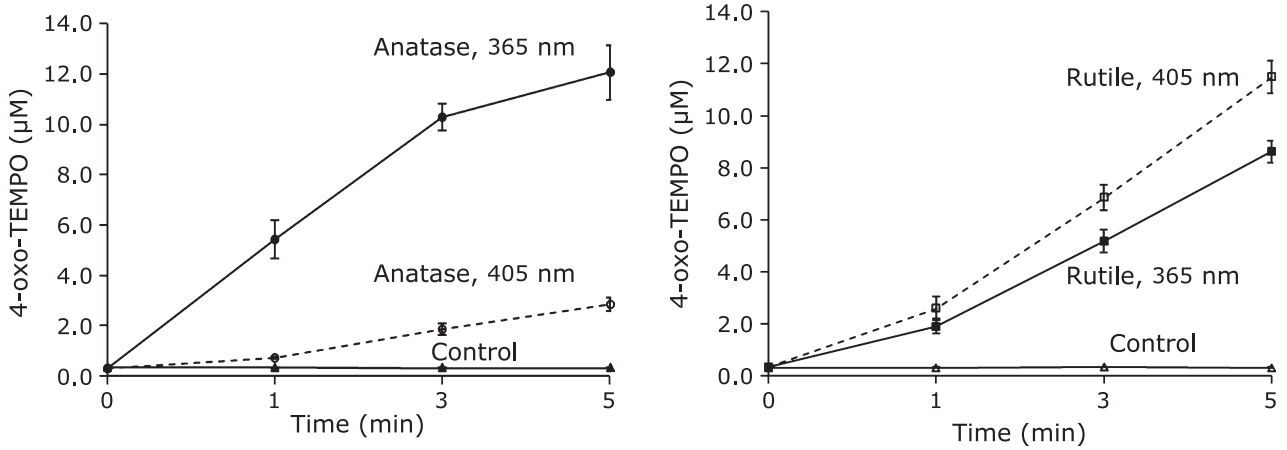


Fig. 2. The formation of 4-oxo-TEMPO upon irradiation of 4-oxo-TMP (40 mM) with TiO_2 (4.0 mg/ml). The data points are the mean values (n = 3) with standard deviation bars.

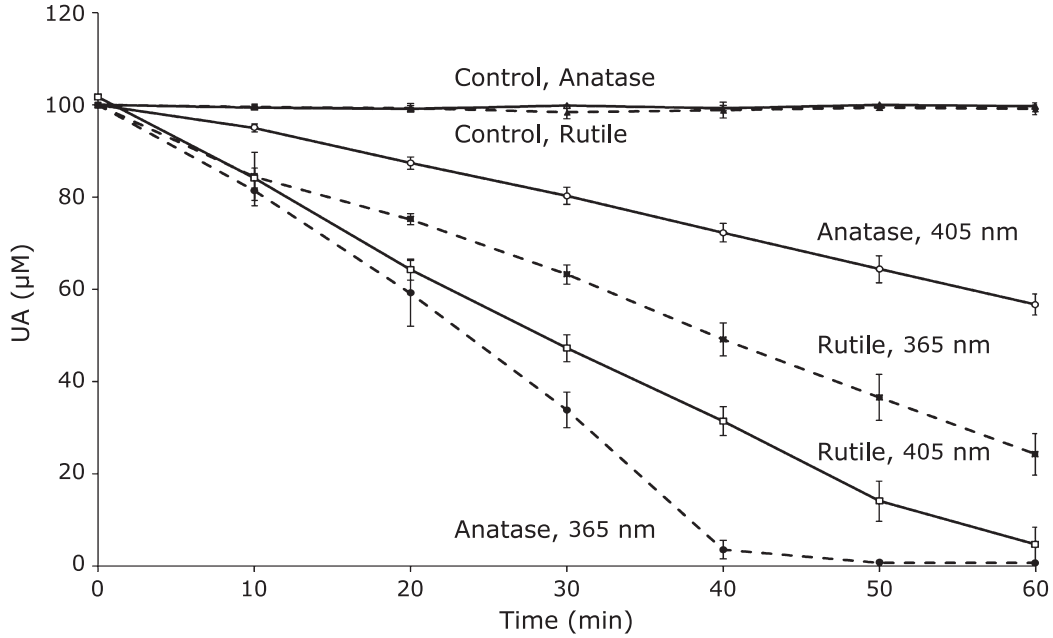


Fig. 3. The photooxidation of uric acid (100 mM) in the presence of TiO_2 (4.0 mg/ml). The values are the means of two independent and reproducible analyses.

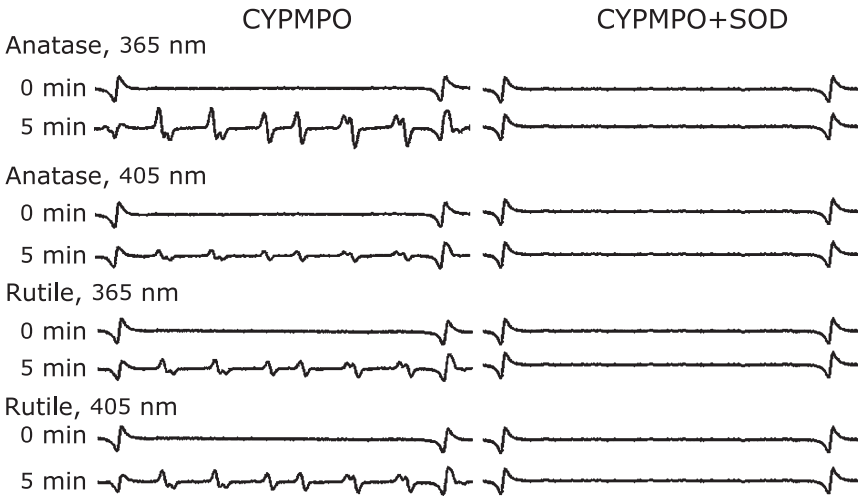


Fig. 4. The formation of OH adducts and their disappearance following the addition of 10 U/ml SOD after the photooxidation of 100 mM CYPMPO in the presence of 0.4 mg/ml TiO_2.

formed, it would abstract the hydrogen atom from the methanol to produce hydroxymethyl radicals ($^{\bullet}CH_2OH$) and methoxyl radicals ($CH_3O^{\bullet}$) (eqs. 2 and 3). Methoxyl radicals give formaldehyde (HCHO) and hydrogen radicals ($H^{\bullet}$) by β-scission (eq. 4). The addition of oxygen to $H^{\bullet}$ and $^{\bullet}CH_2OH$ produces peroxyl radicals ($HOO^{\bullet}$ and $HOCH_2OO^{\bullet}$), respectively (eqs. 5 and 6). These peroxyl radicals should be trapped by BHT, leading to an apparent decrease in the BHT concentration. However, BHT remained unchanged in our study (Fig. 5), thus suggesting that $HO^{\bullet}$ was not formed upon the irradiation of TiO_2 (rutile). This was also the case for the irradiation of the anatase with the 365 and 405 nm lamps (data not shown).

$$HO^{\bullet} + CH_3OH \rightarrow H_2O + {}^{\bullet}CH_2OH \quad (2)$$

$$HO^{\bullet} + CH_3OH \rightarrow H_2O + CH_3O^{\bullet} \quad (3)$$

$$CH_3O^{\bullet} \rightarrow HCHO + H^{\bullet} \quad (4)$$

$$H^{\bullet} + O_2 \rightarrow HOO^{\bullet} \quad (5)$$

$$^{\bullet}CH_2OH + O_2 \rightarrow HOCH_2OO^{\bullet} \quad (6)$$

In conclusion, we measured the rate of $^1O_2$ formation by two methods. The identical results suggest that irradiation of the rutile form of TiO_2 using the 405 nm lamp is the most favorable PDT condition for bacterial killing. We also confirmed that there was no direct $HO^{\bullet}$ formation from the irradiated TiO_2 (rutile). These data support the application of this PDT method for oral bacterial disinfection.

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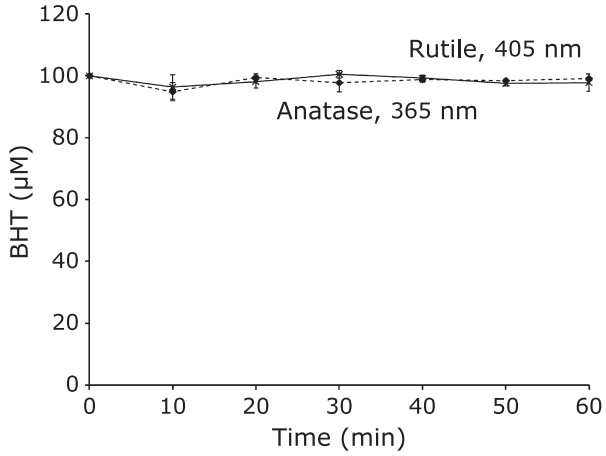

Fig. 5. The oxidation of 2,6-di-tert-butyl-4-methylphenol (BHT, 100 μM) in the presence of TiO_2 (4.0 mg/ml) in aqueous 80% methanol. The values are the means of two independent and reproducible analyses.

Conflict of Interest

No potential conflicts of interest were disclosed.

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Abbreviations

BHT	2,6-di-*tert*-butyl-4-methylphenol
$^{\bullet}CH_2OH$	hydroxymethyl radicals
$CH_3O^{\bullet}$	methoxyl radicals
CYPMPO	5-(2,2-dimethyl-1,3-propoxycyclophosphoryl)-5-methyl-1-pyrroline-*N*-oxide
$H^{\bullet}$	hydrogen radicals
$HO^{\bullet}$	hydroxyl radicals
$^1O_2$	singlet oxygen
$O_2^{\bullet-}$	superoxide
4-oxo-TEMPO	2,2,6,6-tetramethy-4-piperidone-N-oxyl
4-oxo-TMP	2,2,6,6-tetramethyl-4-piperidone
PDT	photodynamic therapy
SOD	superoxide dismutase
TiO_2	titanium dioxide

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