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Photocatalytic bactericidal effect of TiO_2 thin films: dynamic view of the active oxygen species responsible for the effect

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Abstract

The role of active oxygen species in the photocatalytic bactericidal effect was investigated using a thin transparent titanium dioxide (TiO_2) film. The viable number of *Escherichia coli* (*E. coli*) significantly decreased on the illuminated TiO_2 film, and the bactericidal effect was observed even when *E. coli* was separated from the TiO_2 surface with a 50 μm porous membrane. Mannitol, a hydroxyl radical scavenger, inhibited the effect only in the absence of the membrane. In contrast, catalase inhibited the effect in all cases. On the basis of these results, the long-range bactericidal effect of hydrogen peroxide was proposed, together with a cooperative effect due to other oxygen species. © 1997 Elsevier Science S.A.

Keywords: Active oxygen species; *Escherichia coli*; Photocatalytic bactericidal effect; TiO_2 thin film

1. Introduction

Since the discovery of photocatalytic water cleavage on TiO_2 electrodes [1], research work on TiO_2 photocatalysts has been intensively pursued [2,3]. Recently, TiO_2 photocatalysts have been spotlighted as functional materials to aid in the cleaning of the environment [4]. Illuminated TiO_2 photocatalysts decompose organic compounds by oxidation, with holes (h^+) generated in the valence band and with hydroxyl radicals ($OH^\bullet$) produced by the oxidation of water. Such photocatalytic oxidation causes damage to microorganisms, which also consist of organic compounds. Thus photocatalysts are expected to find applications in materials possessing antibacterial functions.

Reports have appeared concerning the bactericidal effects of TiO_2 powder [5–8], often referring to $OH^\bullet$ as the toxic agent. However, there remains some doubt about the actual bactericidal agents because several active oxygen species other than $OH^\bullet$ are generated by photocatalytic reactions, e.g. superoxide anion (O_2^-), perhydroxyl radical ($HOO^\bullet$) and hydrogen peroxide (H_2O_2). These species are better known for their role in biological reactions [9] than in the decomposition of ordinary organic molecules. In addition, bacteria can act as powerful probes to investigate these active oxygen species. Since bacteria are much larger than single molecules, the photocatalytic bactericidal effect necessarily involves long-range interactions between the reactants (bacteria) and the photocatalyst. Such interactions are usually neglected in photocatalytic reactions, which are typically surface reactions for ordinary molecules.

We have successfully produced TiO_2 thin films which are transparent in the visible region, and have demonstrated their high photocatalytic efficiency [10]. The use of a thin film localizes the reaction and also enables modifications to be made conveniently. In this paper, we report the bactericidal effect of TiO_2 thin films with and without modification in order to determine which active oxygen species are responsible for the bactericidal effect and the possible mechanisms.

2. Experimental details

2.1. Materials

Soda-lime glass plates, previously coated with silica thin film (approximately 100 nm), were dipped in titanium isopropoxide solution (Type NTi-500, Nippon Soda) and were slowly pulled from the solution at a fixed rate of 20 cm min^{-1} in dry air. The plates were quickly placed in a furnace and calcined at 500 °C for 1 h. Four such coating steps produced

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a TiO_2 thin film of approximately 0.4 μm on both sides of the glass plate. The thickness was estimated from the interference oscillations in the UV–visible spectra.

Sodium chloride, mannitol, disodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4) were used as received (Wako Pure Chemicals), and the aqueous solutions were sterilized by autoclaving. Catalase (Wako, from bovine liver, 5000–9000 units mg^{-1}) was used as a solution after passing through a membrane filter (Puradisc 25 AS, Whatman, 0.2 μm pore size). Hydrogen peroxide (30%, Wako) was used as received without sterilization. Distilled water and the apparatus, with the exception of the membrane culture insert (see below), were sterilized by autoclaving. TiO_2-coated glass was sterilized in an oven at 70 °C.

2.2. Irradiation and bacterial procedures

Escherichia coli (*E. coli*) cells (IFO 3301 strain) were grown aerobically in 2.5 ml of nutrient broth (''Daigo'', Nippon Seiyaku) at 30 °C for 16–18 h. The cells were centrifuged at 4000 rev min^{-1} and suspended in sterilized water with appropriate dilution.

E. coli cell suspension (150 μl, 2×10^5 cells ml^{-1}, total 3×10^4 cells) was pipetted onto a TiO_2-coated glass plate, spread out to give a liquid film of approximately 1 cm in diameter and placed in an air-tight illumination chamber to prevent drying (Fig. 1(a)). This chamber was illuminated with a 15 W black light (Type FL15 BL-B, National Panasonic); the light intensity, which peaks at around 360 nm, was 1.0 mW cm^{-2}, which was measured using a UV radiometer (UVR-36, Topcon) at the sample position.

After illumination, the cells were removed using a gauze patch and collected in 0.15 mol l^{-1} aqueous sodium chloride solution. This solution was spread onto nutrient agar medium (Standard Method Agar ''Nissui'', Nissui Seiyaku) and incubated for 24 h in order to determine the number of viable cells in terms of colony-forming units.

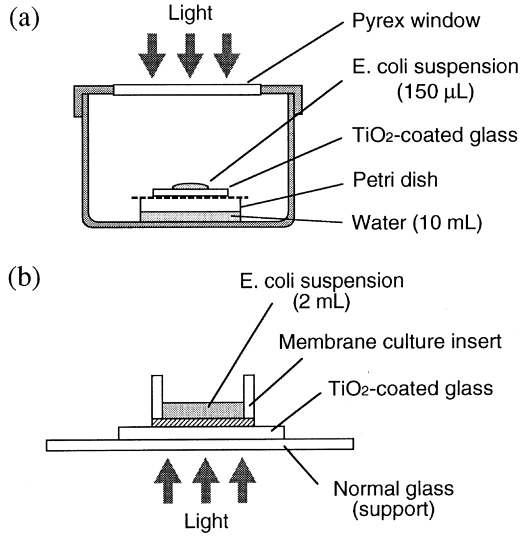

Fig. 1. Systems for illumination: (a) liquid film system; (b) membrane-separated system.

2.3. Separation measurements

For the separation of *E. coli* from the TiO_2 surface, a membrane culture insert (Iwaki Glass) was used, which is in the form of a cylindrical cup, with a hydrophilic PTFE membrane (50 μm thickness, 0.4 μm pore size) forming the bottom, with a capacity of approximately 5 ml (Fig. 1(b)). The culture insert was placed on the TiO_2-coated glass plate; 2 ml of *E. coli* suspension (1.5×10^4 cells ml^{-1}, total 3×10^4 cells) was pipetted into it. This system was illuminated from below. The actual light intensity at the working TiO_2 surface was estimated to be approximately 0.4 mW cm^{-2} because, as it is illuminated from below, the underside TiO_2 layer acts as a UV-attenuating filter. The counting procedure was the same as described above.

2.4. Detection of hydrogen peroxide

Hydrogen peroxide was determined by colorimetry with titanium–4-(2′-pyridylazo)resorcinol reagent (Ti–PAR), which was prepared as described in the literature [11] with PAR obtained from Dojindo Co. Pure water (2 ml) was illuminated (see above). Part of this sample (1 ml) was removed and 1 ml of Ti–PAR reagent and buffer solution was added to it. After warming at 45 °C for 30 min, the absorbance at 508 nm was measured.

3. Results

3.1. Bactericidal effects in the liquid film

The survival ratio for *E. coli* in the liquid film on the TiO_2 film under black light illumination decreases to a negligible level (i.e. essentially complete sterilization) within 1 h (Fig. 2). The efficiency is remarkably higher than that without a TiO_2 film, in which UV light (300–400 nm) causes only 50% sterilization within 4 h. The film in the dark does not affect the survival ratio, indicating that the film itself is not toxic for *E. coli*; this time dependence is almost the same

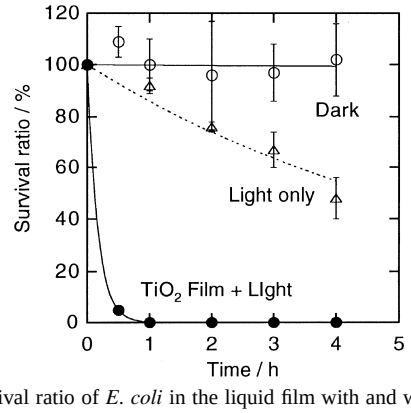

Fig. 2. Survival ratio of *E. coli* in the liquid film with and without a TiO_2 thin film under black light illumination (1.0 mW cm^{-2}) and with a TiO_2 thin film in the dark.

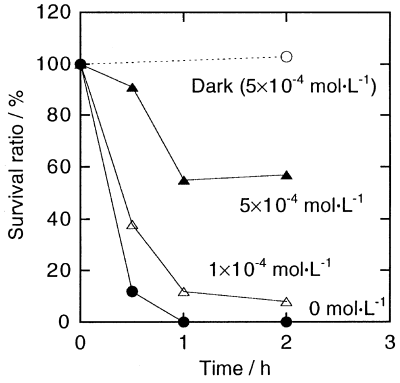

Fig. 3. Effect of mannitol concentration on the survival ratio in the liquid film.

as that on normal glass. Thus the efficient bactericidal effect is due to the photocatalytic reaction of the TiO_2 film.

The addition of mannitol, which is a scavenger for hydroxyl radical ($OH^{\bullet}$), suppresses the level of the bactericidal effect (Fig. 3). Except at higher concentrations (above 5×10^{-3} mol l^{-1}) of mannitol, the survival ratio in the dark is almost the same as that without mannitol, indicating that mannitol itself causes no effect. The survival ratio at a given time significantly increases with increasing mannitol concentration, and the inhibition saturates around 10^{-3} mol l^{-1}.

The effect of pH was examined in order to determine the contribution of reductive photocatalytic pathways; oxygen is reduced to superoxide anion (O_2^-), which is less reactive itself, but produces more highly toxic species, as shown below

$$O_2 \xrightarrow{e^-} O_2^- \underset{-H^+}{\overset{+H^+}{\rightleftarrows}} HOO^{\bullet} \xrightarrow{O_2^-,H^+} H_2O_2 \quad (1)$$

In the present experiments, the initial pH of the *E. coli* suspension is pH 4–5 due to the presence of dissolved carbon dioxide from air. If the pH is adjusted to a higher value, toxic species are more slowly produced from O_2^- [12]. Fig. 4 shows a similar measurement at pH 7.4 using phosphate buffer (Na_2HPO_4–NaH_2PO_4, 8:2); the efficiency of the photocatalytic bactericide is reduced, especially during the early stages of illumination. The concentration of the buffer was 10 mmol l^{-1}, which is sufficiently low to avoid deactivation of the TiO_2 surface due to the adsorption of phosphate ion [13]. The pH effect on $OH^{\bullet}$ formation is expected to be small and opposite for this pH change, so that the effect is due to the inhibition of the reaction in Eq. (1). This result indicates that reductive sites also contribute to the bactericidal effect, in addition to oxidation sites, which produce $OH^{\bullet}$.

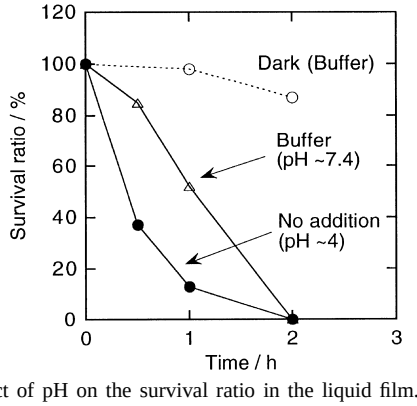

Fig. 4. Effect of pH on the survival ratio in the liquid film. pH 7.4 was obtained using phosphate buffer. The light intensity was attenuated to 0.8 mW cm^{-2} to show the effect clearly.

3.2. Membrane-separated system

The bactericidal effect was also examined in a system in which the *E. coli* suspension was separated from the TiO_2 surface by a porous PTFE membrane. The membrane used for these experiments contains 0.4 μm pores, which prevent *E. coli* from passing through; the actual leak is less than 0.1% of the total cells. Solution phase species can pass through such pores depending on their size; however, radical species are expected to be deactivated before traversing the 50 μm thickness. In spite of the large thickness of the membrane, a photocatalytic bactericidal effect is observed (Fig. 5), with an efficiency similar to that found without the membrane (cylindrical frame only). Although this experiment employs a larger volume of *E. coli* suspension than that in the liquid film, the bactericidal efficiency is almost as great, considering the reduction in the light intensity due to illumination from below.

The effects of mannitol and higher pH were also examined in this system (Fig. 5). Inhibition with mannitol is not observed during the early stages of illumination in spite of the high concentration of mannitol used. On the other hand, the effect of higher pH (pH 7.4) is similar to that observed in the liquid film.

3.3. Contribution of hydrogen peroxide

The effect of catalase, a well-known enzyme which decomposes hydrogen peroxide (H_2O_2) to water and oxygen, was examined in both the liquid film and membrane-separated systems. Inhibition of the photocatalytic bactericidal effect is observed in both systems, indicating the contribution of H_2O_2 to the effect. The time dependence varies in a similar fashion

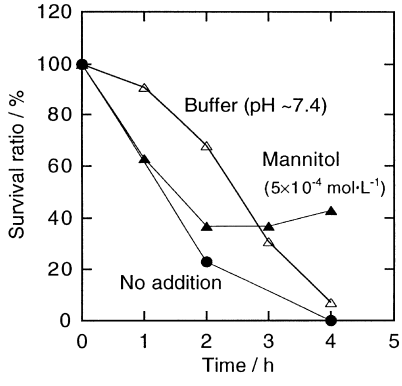

Fig. 5. Survival ratio in the separated system using a porous PTFE membrane. The actual light intensity was 0.4 mW cm^{-2}.

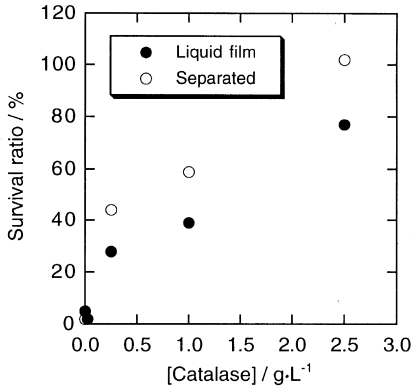

Fig. 6. Dependence of the survival ratio at 2 h (liquid film system) and 3 h (membrane-separated system) on the catalase concentration.

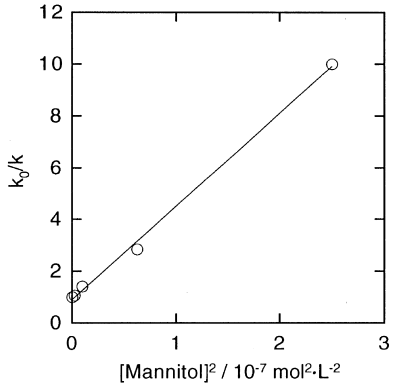

Fig. 7. Concentration effect on mannitol inhibition. k and k_0 are the killing ratios after the first 30 min with and without mannitol respectively.

to that for mannitol inhibition (Fig. 3). Fig. 6 shows the effect of varying catalase concentration on the survival ratio. This result is similar to that reported for the photocatalytic killing of cancer cells [14]. Other inhibitory effects are negligible, e.g. UV attenuation due to the absorption of catalase and the inhibition of the bactericidal effect by UV light.

The production of H_2O_2 was detected colorimetrically with Ti–PAR. In the membrane-separated system, approximately 2×10^{-7} mol l^{-1} of H_2O_2 is observed in pure water in contact with the TiO_2 film after 2 h illumination.

4. Discussion

4.1. Bactericidal agent

Hydroxyl radical ($OH^{\bullet}$) reacts with various organic compounds at essentially a diffusion-limited rate [15] and is considered to be the primary agent in the photocatalytic bactericidal effect. A similar conclusion may be reached from the result that the photocatalytic bactericide in the liquid film is strongly inhibited by mannitol. In the light of the other results reported here, however, this scheme should be modified.

The contribution of hydrogen peroxide (H_2O_2) to the effect should be considered, because inhibition by catalase is observed. Most importantly, in the membrane-separated system, catalase has a significant effect, whereas that due to mannitol is small. These results suggest that the main bactericidal agent is not $OH^{\bullet}$ but H_2O_2 in this system. H_2O_2 is thought to be produced at both oxidation and reduction sites in photocatalysis, via the coupling of $OH^{\bullet}$ at the former and the reduction or disproportionation of O_2^- at the latter. The latter is also confirmed by the effect of pH, which is significant even in the membrane-separated system.

Catalase is effective even in the liquid film system. The major difference between the liquid film and the membrane-separated systems is the magnitude of mannitol inhibition, which is significant in the liquid film system. However, this does not necessarily provide evidence for the contribution of $OH^{\bullet}$, because it is also possible that mannitol inhibits the formation of H_2O_2 via $OH^{\bullet}$, which is likely to be observed in the liquid film. The effect of concentration on the mannitol inhibition is suggestive (Fig. 7). Here, the inverse of the relative killing ratio after the first 30 min (k_0/k) linearly increases with the square of the mannitol concentration. This relationship seems to support the above view, i.e. two molecules of mannitol are required to inhibit H_2O_2 formation from two molecules of $OH^{\bullet}$.

There are several reports suggesting that $OH^{\bullet}$ cannot permeate through cell membranes [16,17], but H_2O_2 can [18]. In fact, it is conceivable that the direct attack of $OH^{\bullet}$ and the destruction of the cell membrane of *E. coli* may be difficult, as discussed below.

4.2. Mobility of hydroxyl radical

The high reactivity of $OH^{\bullet}$ is consistent with a shorter lifetime and a smaller effective mobility; this aspect must be discussed prior to the permeability of the cell membrane. The steady state concentration and half-life of $OH^{\bullet}$ generated in the present photocatalytic system were estimated for the case in which there is no reactant other than water. The concentration changes of $OH^{\bullet}$ and O_2^- are expressed in Eq. (2) and Eq. (3) respectively

$$\frac{d}{dt}[OH^{\bullet}] = I_a\Phi - k_D[OH^{\bullet}]^2 - k_D[OH^{\bullet}][O_2^-] \quad (2)$$

$$\frac{d}{dt}[O_2^-] = \alpha I_a\Phi - k_D[OH^{\bullet}][O_2^-] \quad (3)$$

where I_a is the number of light quanta absorbed per second, Φ is the quantum yield (assumed to be the same for $OH^{\bullet}$ and O_2^-), k_D is the diffusion-limited rate constant and α is the mole fraction of O_2^- in the equilibrium between O_2^- and $OOH^{\bullet}$, dependent on the pH (Eq. (1)).

In Eq. (3), other processes which remove $OH^{\bullet}$ and O_2^- were omitted because their rate constants are much lower than k_D. The steady state concentration of $OH^{\bullet}$ near the TiO_2 surface ($[OH^{\bullet}]_S$) can be expressed as follows

$$[OH^{\bullet}]_S = \sqrt{\frac{(1-\alpha)I_a\Phi}{k_D}} \quad (4)$$

Table 1
Parameters of $OH^{\bullet}$

System	$[OH^{\bullet}]_S$ (mol l^{-1}) [a]	Half-life (μs) [b]	Half-length (μm) [c]
Liquid film	3.2×10^{-7}	310	1.7
Membrane-separated	2.3×10^{-7}	440	2.0

[a] See text.
[b] Half-life of $OH^{\bullet}$ assuming second-order kinetics and neglecting O_2^-: $\tau_{1/2}=1/k_D[OH^{\bullet}]_S$.
[c] The length $OH^{\bullet}$ can travel within its half-life ($2\sqrt{D\tau_{1/2}}$), where D is the diffusion coefficient for $OH^{\bullet}$ assumed to be $2.3\times10^{-9}\,m^2\,s^{-1}$ (self-diffusion coefficient of water).

Under the present conditions, I_a was estimated to be 0.030 mol l^{-1} s^{-1} and 0.016 mol l^{-1} s^{-1} for the liquid film and membrane-separated systems respectively. α is 0.173 at pH 4, based on the pK_a value of 4.68 for $OOH^{\bullet}$. With the assumption $\Phi=0.04$ [19] and $k_D=1\times10^{10}$ mol l^{-1} s^{-1}, $[OH^{\bullet}]_S$ and other values were estimated and are listed in Table 1. The half-life of $OH^{\bullet}$ released to bulk water was approximately estimated to be that due to second-order kinetics, in which the presence of O_2^-, at a concentration of about 20% of $[OH^{\bullet}]_S$, was omitted for convenience. The half-length for $OH^{\bullet}$, corresponding to its half-life, was limited to a few micrometres, and this value did not vary significantly even if the assumptions were somewhat altered. In addition, the experimental value of the H_2O_2 concentration, 2×10^{-7} mol l^{-1}, was of the same order as $[OH^{\bullet}]_S$ estimated for the membrane-separated system, $OH^{\bullet}$ being one of the sources of H_2O_2.

Thus it is virtually impossible for $OH^{\bullet}$ to traverse the 50 μm of separating membrane. Moreover, the half-length estimated seems insufficient even for attack of the *E. coli* cells directly, unless they are in very close contact with the TiO_2 surface, which is not likely since the recovery of cells on sampling is almost 100%.

4.3. Hydrogen peroxide

The generation of H_2O_2 has often been reported in aqueous systems containing TiO_2 photocatalysts [20–22]. The bactericidal action of H_2O_2 is also well known. It has two modes of action: mode I occurring at H_2O_2 concentrations below 2 mmol l^{-1}, and mode II occurring at concentrations greater than 10 mmol l^{-1} [23,24]. Mode I killing occurs due to unknown species related to metabolism, and mode II killing occurs due to $OH^{\bullet}$ resulting from the Fenton reaction. In this study, 2×10^{-7} mol l^{-1} of H_2O_2 was observed in the membrane-separated system, and this concentration falls in the range for mode I killing. However, 10^{-7}–10^{-4} mol l^{-1} of authentic H_2O_2 solution exhibits almost no killing effect; this result is also expected from the reported value [24]: 0.01% h^{-1}. These results are apparently inconsistent with the main suggestion in this paper.

This may be explained by the cooperative effect of other oxygen species. It is unlikely that there is an effect due to the direct photolysis of H_2O_2, because the UV light used in this work is not absorbed by H_2O_2. The key intermediate is proposed to be O_2^-, which is produced from illuminated *E. coli* itself or TiO_2. The bactericidal effect of near-UV light (300–400 nm) is considered to be caused by O_2^- and/or singlet oxygen ($^1O_2^*$) generated by photosensitization, in which the sensitizer is a cell component possessing a large π-conjugated system, e.g. riboflavin [25]. It is well known that O_2^- is involved in the Harber–Weiss reaction [26]

$$H_2O_2 + O_2^- \rightarrow OH^{\bullet} + OH^- + O_2 \quad (5)$$

If this occurs inside a cell, the damage is expected to be amplified. It has been reported that a mutant of *E. coli* lacking superoxide dismutase is sensitive to mode I killing with H_2O_2 [27]. This result seems to support our hypothesis. H_2O_2 addition under black light illumination was tested; however, the killing effect was scarcely enhanced, and this result was still different from that given in Fig. 2. Another type of species arising from the photocatalytic reaction is apparently needed, but the verification of this is highly complicated. In the membrane-separated system, the local concentration of H_2O_2 near the membrane may also be considerable. Further investigation is needed to clarify this.

5. Conclusions

In the photocatalytic bactericidal effect of *E. coli* on illuminated TiO_2 films, it was confirmed that both oxidation and reduction sites contribute, corresponding to $OH^{\bullet}$ and O_2^- production respectively. However, it is proposed that the actual lethal agent is H_2O_2, subsequently produced from $OH^{\bullet}$ and O_2^-, particularly in the long-range bactericidal effect. The concentration of H_2O_2 produced photocatalytically is low, and thus a cooperative effect with other oxygen species is postulated.

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References

[1] A. Fujishima, K. Honda, Nature 37 (1972) 238.
[2] A.L. Linsebigler, G. Lu, J.T. Yates Jr., Chem. Rev. 95 (1995) 735.
[3] M.R. Hoffmann, S.T. Martin, W. Choi, D.W. Bahnemann, Chem. Rev. 95 (1995) 69.
[4] D.F. Ollis, E. Pelizetti, N. Serpone, Environ. Sci. Technol. 25 (1991) 1523.

[5] T. Matsunaga, R. Tomoda, T. Nakajima, H. Wake, FEMS Microbiol. Lett. 22 (1985) 211.
[6] J.C. Ireland, P. Klostermann, W. Rice, R.M. Clark, Appl. Environ. Microbiol. 59 (1993) 1668.
[7] C. Wei, W.-Y. Lin, Z. Zainal, N.E. Williams, K. Zhu, A.P. Kruzic, R.L. Smith, K. Rajeshwar, Environ. Sci. Technol. 28 (1994) 934.
[8] R.J. Watts, S. Kong, M.P. Orr, G.C. Miller, B.E. Henry, Water Res. 29 (1995) 95.
[9] B. Halliwell, J.M.C. Gutteridge, Biochem. J. 219 (1984) 1.
[10] N. Neghishi, T. Iyoda, K. Hashimoto, A. Fujishima, Chem. Lett. (1995) 841.
[11] C. Matsubara, T. Iwamoto, Y. Nishikawa, K. Takamura, S. Yano, S. Yoshikawa, J. Chem. Soc., Dalton Trans. (1985) 81.
[12] S. Marklund, J. Biol. Chem. 251 (1976) 7504.
[13] M. Abdullah, G.K.-C. Low, R.W. Matthews, J. Phys. Chem. 94 (1990) 6820.
[14] R. Cai, K. Hashimoto, Y. Kubota, A. Fujishima, Chem. Lett. (1992) 427.
[15] K.U. Ingold, in: J.K. Kochi (Ed.), Free Radicals, Wiley-Interscience, New York, 1973, p. 37.
[16] G. Strul, A.A. Frimer, L. Weiner, J. Chem. Soc., Perkin Trans. (1993) 2057.
[17] I. Ueno, M. Kohno, K. Mitsuta, Y. Mizuta, S. Kanegasaki, J. Biochem. 105 (1989) 905.
[18] M. Takahashi, K. Asada, Arch. Biochem. Biophys. 226 (1983) 558.
[19] L. Sun, J.R. Bolton, J. Phys. Chem. 100 (1996) 4127.
[20] M.V. Rao, K. Rajeshwar, V.R. Pai Verneker, J. DuBow, J. Phys. Chem. 84 (1980) 1987.
[21] R. Cai, K. Hashimoto, A. Fujishima, Y. Kubota, J. Electroanal. Chem. 326 (1992) 345.
[22] H. Sakai, R. Baba, K. Hashimoto, A. Fujishima, A. Heller, J. Phys. Chem. 99 (1995) 11 896.
[23] J.A. Imlay, S. Linn, J. Bacteriol. 166 (1986) 519.
[24] G. Brandi, F. Cattabeni, A. Albano, O. Cantoni, Free Rad. Res. Commun. 6 (1989) 47.
[25] A. Hollaender, J. Bacteriol. 46 (1943) 531.
[26] M. Fujihira, Y. Satoh, T. Osa, Bull. Chem. Soc. Jpn. 55 (1982) 666.
[27] J.A. Imlay, S. Linn, J. Bacteriol. 169 (1987) 2967.