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Visible-Light Bactericidal Effect of Silica Gel-supported Porphyrinatoantimony(V) Catalyst on *Legionella* Species Occurring in the Living Environmental Fields

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In order to develop a bactericidal agent which can operate under visible-light irradiation, silica gel-supported dihydroxo(tetraphenylporphyrinato)antimony(V) catalyst (SbTPP/SiO₂) was prepared. Experiments to reduce concentrations of *Legionella* species were performed using a cylindrical SbTPP/SiO₂-photocatalytic bactericidal apparatus in a cooling tower which held 800L of water. After 10 days, the concentrations of *Legionella* species were reduced to less than the detection limit, and these levels were kept until the irradiation was stopped. Also, a photocatalytic bactericidal experiment was conducted with a fountain that was filled with 13m³ of water. The concentrations of *Legionella* species were reduced to less than the detection limit 12 days after the SbTPP/SiO₂ catalyst was installed in the fountain receiving sunlight irradiation. The concentrations of *Legionella* species were kept at less than 30 CFU/100ml for 3 months until the catalyst was removed from the fountain. Thus, visible-light irradiation of the SbTPP/SiO₂ catalyst induced a remarkable bactericidal activity against *Legionella* species in the living environment.

Key words : Bactericidal effect/*Legionella* species/Dihydroxo(tetraphenylporphyrinato)antimony(V) complex/SiO₂ support/Living environmental fields.

INTRODUCTION

Legionella pneumophila has drawn much concern since the first outbreak of Legionnaires' disease in Philadelphia in 1976 (Chang and Yu, 1996). Recently, large outbreaks of Legionnaires' disease have been reported in Portugal, Netherlands, and Spain (Correia et al., 2001; Den Boer et al., 2002; Fernández et al., 2002; Lettinga et al., 2002). The

natural habitats for *L. pneumophila* are a wide range of aquatic bodies including lakes, streams, and artificially constructed aquatic reservoirs such as fountains and cooling-towers.

On the other hand, the photocatalytic treatments have received much attention as an environmentally friendly process to degrade organic compounds in contaminated water (Legrini et al., 1993). From the standpoint of developing a visible-light driven photocatalyst we have focused on the high-valent metalloporphyrins complexes that have a strong absorption in the visible region. We have previously

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reported on the silica gel-supported dihydroxo (tetraphenyl-porphyrinato)antimony(V) (SbTPP/SiO₂) which was effective for the photochemical dechlorination of 4-chlorophenol (Shiragami et al., 2003). Moreover, our recent investigation has elucidated that SbTPP/SiO₂ works as bactericidal agent against *E. coli* in aqueous solution under visible light irradiation (Yokoi et al., 2003). In this study, therefore, we will report on the photocatalytic bactericidal activity of the SbTPP/SiO₂ to reduce *Legionella* concentrations and our practical experiments involving a cooling tower and public fountain which can be found in our living environment.

MATERIALS AND METHODS

Instruments

For the elemental analysis of the catalysts, the catalysts (2g) were calcined at 950°C for 2h and put into a mixture of water (2ml) and HF solution (46%, 10ml) and heated at 200°C to remove Si component as SiF₄. The residual components were dissolved in hot HNO₃ (1 mol/L, 5 ml) and subjected to the elemental analysis of Al and Sb on a Shimadzu AA-6200 atomic absorption spectrometer and a Shimadzu ICPS-7500 inductively coupled plasma (ICP) spectrometer, respectively. The atomic absorption analysis of other metals such as Na, Ca, Fe and Mg metals was performed after the addition of aqueous HCl solution of La₂O₃ (5wt%) as the matrix modifier. Microscopic spectroscopy of the catalysts was performed on an Olympus FV-300 confocal laser scanning microscope (CLSM) equipped with spectrophotometer (STFL 250, Seki Technotron) linked to CLSM with an optical fiber. Light intensity was measured at the wavelength of 420nm by a power meter (TQ8210, Advantest).

Preparation of the photocatalyst

Dihydroxo(tetraphenylporphyrinato)antimony (V) bromide, [SbTPP(OH)₂]Br, was prepared according to the literature (Andou et al., 2001; Shiragami et al., 1996; Takagi et al., 1997). Into toluene solution (400

ml) of [SbTPP(OH)₂]Br (170mg), silica-gel powder (p-SiO₂; 30g, 300mesh, 0.04mmf, 429m²/g, BW300, Fuji Silysia Chemical Ltd., Japan) was added and then refluxed for 18h (Shiragami et al., 2002). The treated silica-gel was filtered, washed with acetone (100ml), and then dried under reduced pressure to give the p-SiO₂-supported [SbTPP(OH)₂]⁺ catalyst (SbTPP/p-SiO₂) where the content of [SbTPP(OH)₂]⁺Br⁻ chromophore was 0.87wt% (Fig. 1).

Into MeOH-toluene solution (1:4 v/v, 500ml) of [SbTPP(OH)₂]Br (35mg), silica gel beads (b-SiO₂; 70g, 1.7-4.0mmf, 306m²/g, CARIAC T Q-10, Fuji Silysia Chemical Ltd. Japan) were added and then the solution was made to stand for 18h. The MeOH was removed by distillation from the solution. The treated silica gel was filtered, dried at 70°C, washed with acetone (200ml), and then dried at 70°C to give the b-SiO₂-supported [SbTPP(OH)₂]⁺ catalyst (SbTPP/b-SiO₂) where the content of [SbTPP(OH)₂]⁺Br⁻ chromophore was 0.05wt%. The prepared catalyst was identified by the observation of the characteristic fluorescence of SbTPP chromophore by CLSM analysis (Fig. 2).

Photocatalytic sterilization in L-type glass tubes

The bactericidal activities of the SbTPP/p-SiO₂ on *L. pneumophila* were examined in an L-type glass tube in similar manner to the method reported for *E. coli* (Yokoi et al., 2003). *L. pneumophila* was isolated from a cooling tower and identified to be Type I by the PCR method using a leg primer. The phosphate buffer (9ml, 100mmol/L, pH7.0), the cell suspension of *L. pneumophila* (1.0 ml, ca. 10⁶ CFU/100ml), and SbTPP/p-SiO₂ (10mg) were poured into the L-type glass tube (length 18cm, diameter 1.5cm). The L-type glass tube was set on a reciprocal shaker and irradiated by two fluorescent lamps, 15W each, which were set above the shaker. Light intensity on the surface of the L-type glass tubes was 21W/m² and the reaction temperature was kept constant at 30°C.

A portion (0.1ml) of the reaction mixture was directly plated on a selective medium for *Legionella*

FIG. 1. Preparation of SbTPP/p- or b-SiO₂ from the reaction of [SbTPP(OH)₂]Br with SiO₂.

FIG. 2. The imaging and fluorescence spectra of SbTPP/b-SiO₂ measured on CLSM.

species: WYO α agar medium (Eiken Chemicals Co., Ltd, Japan) consisting of 3g of glycine, 5mg of vancomycin, 10⁶ IU of polymixin B, and 80mg of amphotericin B. The colonies of *L. pneumophila*, which appeared after incubation for 7 d at 36°C were counted in three replicate plates.

Practical experiments in a cooling-tower using a cylindrical bactericidal apparatus

The bactericidal experiment was performed in a cooling tower which was set in a hospital in Miyazaki city, using a cylindrical apparatus (20cm ϕ $\times$ 50cm, Fig. 3A) consisting of 7 fluorescent lamps (18W, 4.3 cm ϕ $\times$ 50cm) and the SbTPP/g-SiO₂ catalyst (4.0kg), as shown in Fig. 3B. Water in the holder (800 L) of the cooling tower was fed to the cylindrical vessel by a pump at 28L/min and then the treated water was returned to the holder. The average retention time was calculated to be 26s. In the cylindrical vessel, the SbTPP/b-SiO₂ catalyst was irradiated by visible-light emitted from fluorescent lamps at ambient temperature. The sampling was carried out at the outlet of the apparatus at 3-7 d intervals. At same time, the atmospheric temperatures were recorded as the average values of the highest temperature of Miyazaki city during three days of the sampling day and two days before the sampling day. Viable cell numbers of bacteria that were mostly presumed to be *Legionella* species were determined as follows. The sample water (1000ml) was filtrated by membrane filter (0.45 μ m HA, Millipore) under reduced pressure. Into the vessel (100ml) containing the microbes adhering to the membrane filter, an aqueous solution (5ml) was added and then the vessel was shaken vigorously. Additional aqueous saturated KCl solution (5ml; pH2.2) containing 0.2M HCl was added to the vessel and which then was shaken and made to stand exactly for 20min at room temperature to give the prepared solution. A portion (0.1ml) of the

prepared solution was plated on a WYO α agar medium and incubated for 7 d at 36°C. Wet, smooth, and bluish-white colonies were counted on three replicate plates.

Practical experiments in a fountain using a leaf-type bactericidal apparatus

Practical experiments were performed in a public fountain that was filled with 13m³ of water in a period from November 8, 2002 to October 29, 2003 in Miyazaki City. Bactericidal effects were examined using a leaf-type photocatalytic bactericidal apparatus (20cm ϕ $\times$ 2cm; Fig. 3C) containing the SbTPP/b-SiO₂ catalyst (80g) under sunlight irradiation, as shown in Fig. 3D. The samplings were arbitrarily carried out at the pool of the fountain at 5-7 d intervals. At same time, the atmospheric temperatures were recorded as mentioned above. Determination of viable

FIG. 3. A cylindrical type photo-bactericidal apparatus (A) was set in the cooling-tower (B). A leaf-type photo-bactericidal apparatus (C) was installed in the fountain (D).

cell numbers of *Legionella* species was carried out in the same manner as in the practical experiments in the cooling-tower.

RESULTS AND DISCUSSION

Photocatalytic sterilization in L-type glass tube

The bactericidal effect of SbTPP/SiO₂ on *Legionella* species in L-type glass tubes was carried out under irradiation by fluorescent lamps. Figure 4 shows time courses of survival ratio of *Legionella* species in L-type glass tubes where the SbTPP/p-SiO₂ photocatalyst (10mg) was irradiated by fluorescent lamps in the presence of the cell suspensions

FIG. 4. Bactericidal effect of SbTPP/p-SiO₂ catalyst on *L. pneumophila* under irradiation by fluorescent lamp in the presence of the catalyst (●), under the irradiation in the absence of the catalyst (○), under dark reaction in the presence of the catalyst (▲), and under dark reaction in the absence of the catalyst (△).

FIG. 5. Time-course plots of the amounts of *Legionella* species in the cooling-tower along with the atmospheric temperature (●). The cylindrical photo-bactericidal apparatus was operated during October 1 to October 21, 2002. Conditions; catalyst: 4 kg, contents of water: ca. 800 L, flow rate: 28 L/min, and the average retention time: 26 s.

(10ml). Upon irradiation for 60min in the presence of the SbTPP/p-SiO₂, the concentration of *Legionella* species apparently decreased from the initial concentration (6.4×10^5 CFU/100ml) to 4×10^3 CFU/100ml: its survival ratio was 0.6%. In the control experiments under irradiation in the absence of SbTPP/p-SiO₂, under dark conditions in the presence of SbTPP/p-SiO₂, and under dark conditions in the absence of SbTPP/p-SiO₂, nearly the initial concentration of *Legionella* species was kept. Thus, it is confirmed that SbTPP/p-SiO₂ has the photocatalytic activity to reduce concentrations of *Legionella* species.

Practical experiments in the cooling-tower

The bactericidal effect of SbTPP/b-SiO₂ was substantiated in the practical experiments at a cooling-tower using a cylindrical photocatalytic bactericidal apparatus (Fig. 3A). Under the conditions without any bactericidal treatments, *Legionella* species were found in the range of 20 to 139CFU/100ml in the holder of the cooling tower, as shown in Fig. 5. After the bactericidal apparatus was operated for 4 days, the concentration of *Legionella* species was reduced to 22CFU/100ml. Further operation reduced the concentrations of *Legionella* species to reach less than the detection limit, and these levels were kept until the irradiation was stopped. Seven days after the irradiation was stopped, detectable amounts of *Legionella* species appeared. Thus, bactericidal effects of SbTPP/b-SiO₂ were practically confirmed in practical experiments involving cooling tower.

Practical experiments in the fountain

The preliminary concentrations of *Legionella* species naturally occurring in a public fountain were measured for 7 months from November, 2002 to May, 2003 under the conditions without any bactericidal treatments. Figure 6A showed the time-course plots of the concentrations of *Legionella* species, which were strongly dependent on atmospheric temperature. As the atmospheric temperature increased, the concentrations of *Legionella* species also increased, as shown in Fig. 7. Concentrations of *Legionella* species exceeded 500CFU/100ml in some samples when the atmospheric temperature went over 25°C.

Practical bactericidal experiments were performed using a leaf-type photo bactericidal apparatus containing 80g of SbTPP/b-SiO₂, which was set into the fountain under sunlight irradiation during the period from May 28, 2003 to August 25, 2003. The concentration of *Legionella* species were reduced to less than the detection limit, 12 d after the leaf-type apparatus was installed to the fountain.

The concentrations of *Legionella* species were

continuously kept at less than 30 CFU/100ml until the leaf-type apparatus was removed from the fountain on August 25 (Fig. 6B). After the removal of the leaf-type apparatus, the concentrations of *Legionella* species were gradually increased to reach 100 CFU/100ml, the environmental quality standard, 42 d after the removal of the leaf-type apparatus, as shown in Fig. 6B. In these practical experiments, it is noteworthy that 80g of SbTPP/b-SiO₂ catalyst, i.e. 40mg of [SbTPP(OH)₂]⁺Br⁻ chromophore, kept the concentration of *Legionella* species in 13m³ of water below 100CFU/100ml for 120 d.

Analysis of the catalyst

Elemental analysis of the catalyst before and after its use was performed by atomic absorption and ICP (Table 1). Before being used, the Sb content in SbTPP/b-SiO₂ was measured to be 80ppm, which showed good agreement with the Sb content (72ppm) calculated for the 0.05wt% of SbTPP content in the catalyst. Contents of metals other than Sb in the catalyst were as follows: 67 (Na), 6 (Mg), 29 (Al), 12 (Ca), and 0.2ppm (Fe). After being used in the practical experiment for 3 months in the fountain, the Sb content decreased from 80ppm to 17ppm. On

FIG. 6. (A) Survey of *Legionella* species found in the fountain from November 26, 2002 to May 26, 2003. (B) the bactericidal experiment of *Legionella* species was performed using a leaf-type photo-bactericidal apparatus containing SbTPP/SiO₂ (80g) under sunlight irradiation from May 26 to Aug. 22, 2003.

FIG. 7. Dependence of amounts of *Legionella* species naturally occurring in the fountain on the atmospheric temperature.

the other hand, Na, Mg, Al, and Ca largely increased, resulting in the occurrence of the ion-absorption on SiO₂. Moreover, the analysis of the SbTPP/b-SiO₂ catalyst used for the practical experiment in the fountain was performed by CLSM (Fig. 8). It was found that the fluorescence come from the surface of the catalyst kept a shape similar to that of the original catalyst, but the intensity was weaker compared with the original spectra of SbTPP/b-SiO₂. On the other hand, the fluorescence from the inside of the catalyst maintained its original intensity. Therefore, it is suggested that [SbTPP(OH)₂]⁺ chromophore was eliminated from the surface of catalyst. Irradiation of fluorescent light on the SbTPP/b-SiO₂ catalyst in deionized water did not entirely cause the spectral change and decrease of Sb-amounts. Therefore, it is strongly suggested that the cationic [SbTPP(OH)₂]⁺ chromophore was exchanged with alkali metal ions in the bulk water on the surface of catalyst under irradiation.

TABLE 1. Elemental analysis of SbTPP/SiO₂.^a

Item	Element ^b					
	Na	Mg	Al	Ca	Fe	Sb
Before Use	67	6	29	12	0.2	80 ^c
After Use ^d	208	79	89	316	0.5	13

^a SbTPP/SiO₂ photo-catalyst of which the SbTPP content was 0.05 wt%.

^b Content of elements in ppm.

^c This value showed good agreement to the Sb content (72ppm) of 0.05wt% of SbTPP.

^d The SbTPP/b-SiO₂ catalyst after the practical experiment in the fountain for 3 months.

Water of the fountain involved the following minerals: Al= 2, Na=3, Ca= 18, Fe=0.5, Mg=9, Sb<0.04 ppm.

FIG. 8. CLSM fluorescence spectra of SbTPP/*b*-SiO₂ before (solid line) and after use for the practical experiment in the fountain: Spectra of the surface (broken line) of the catalyst after use.

In conclusion, the SbTPP/*b*-SiO₂ catalyst operated effectively to reduce *Legionella* concentrations under visible-light irradiation in the living environments. It is well known that the photocatalytic process by titanium dioxide (TiO₂) has been widely applied to the disinfection and sterilization (Kühn et al., 2003; Matsunaga et al., 1985; Saito et al., 1992; Theurich et al., 1996) as well as treatment of wastewater (Legrini et al., 1993). However, TiO₂ has only weak absorption in the visible region. It is, therefore, expected that the present visible-light bactericidal technique by the SbTPP/*b*-SiO₂ catalyst will be one of the powerful methods to sterilize aquatic bodies of harmful microbes.

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