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Bactericidal Activity of Copper-containing Sulfur-doped TiO_2 against *Staphylococcus aureus* under Visible-light Illumination

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The bactericidal activity of a sulfur-doped titanium dioxide (TiO_2) thin film was investigated against *Staphylococcus aureus* under visible-light illumination. The bactericidal activity of sulfur-doped TiO_2 was higher than undoped TiO_2. Addition of copper, silver, and nickel to TiO_2, increased antibacterial activity, with 4–10% copper addition yielding the most activity. The bactericidal activity of copper-containing sulfur-doped TiO_2 under daylight-like conditions was effective. These results suggested that copper-containing sulfur-doped TiO_2 may be applicable for environmental uses.

Key words: Sulfur-doped TiO_2 plus copper metal, bactericidal activity, visible-light illumination, *Staphylococcus aureus*

1. Introduction

Staphylococcus aureus is a versatile pathogenic bacterium capable of rapidly developing or acquiring multiple antibiotic resistances, and is now recognized as a worldwide health problem[9]. *S. aureus* is responsible for a wide spectrum of human and animal diseases, ranging from benign skin infections to severe diseases, such as arthritis, osteomyelitis, endocarditis or fatal sepsis[17]. In the hospital setting, the most common mode of transmission of resistant *S. aureus* is close contact with infected persons or with health-care workers with contaminated hands or clothing[6]. In addition, *S. aureus* is spread by airborne infection[7]. *S. aureus* has been a significant problem not only in hospitals but also common house[13]. Therefore, a new technology to sterilize *S. aureus* is necessary.

Disinfectants are antimicrobial agents used extensively in hospitals and other health care settings for a variety of topical and hard-surface applications. They are essential for infection control and aid in the prevention of nosocomial infections[16]. Compared to antibiotics, which provide comparatively selective activity against microorganisms, disinfectants typically have a broader antibacterial spectrum[20] and are usually used on non-living surfaces[31]. A wide variety of active chemical agents exhibit bactericidal activities. Some of the most widely used, including alcohols, iodine, and chlorine, have been employed for a comparatively long time in disinfection and preservation[20]. Titanium dioxide (TiO_2) is one such disinfectant.

The photocatalytic antimicrobial activities of TiO_2 have been well documented[3,18,19,22,23,26,30]. The first published observations of the biocidal effects of photocatalytic TiO_2 were made using *Escherichia coli*[19] and have since been extended to include a wide spectrum of organisms including viruses, other bacteria, fungi, algae, and cancer cells[3,18]. In the last decade, TiO_2 has been widely utilized as a self-cleaning and self-sterilizing material for coating many clinical tools used in hospitals and sanitary ware, including food tableware and cooking ware[11]. However, the TiO_2 photocatalyst is effective only upon irradiation by UV light at levels that would induce serious damage to human cells. This greatly restricts the potential application of TiO_2 substrates for use in many work and home environments. Recently, anion-doped TiO_2-based photocatalysts have been identified, which are activated by visible light, offering the potential to overcome this limitation. Sakthuvel *et al.*[27] investigated daylight-mediated photocatalysis of carbon-doped TiO_2. Asahi *et al.*[1] reported theoretical calculation of the band structure of nitrogen-doped TiO_2 and its visible light-photocatalyzed-degradation of acetaldehyde and methylene blue. According to Wong *et al.*[35], nitrogen-doped TiO_2 thin film has superior visible-light-induced bactericidal activity against *E. coli* relative to pure TiO_2 thin film and carbon-doped TiO_2 thin film. Recently, Ohno *et al.*[25] succeeded in synthesizing sulfur-doped TiO_2, and showed that it strongly absorbed visible light, degraded methylene blue and 2-propanol in aqueous solution, and partially oxidized adamantine when irradiated with light at wavelengths greater than 440 nm. However, little is known about the bactericidal activity of sulfur-doped TiO_2.

In the present study, to our knowledge, we report that sulfur-doped TiO_2 exhibits bactericidal effects towards *S. aureus* in water under visible light. Further, we report that addition of copper, nickel, and silver to sulfur-doped TiO_2 films increased such activity.

2. Materials and Methods

2.1. Preparation of substrates coated with TiO_2, sulfur-doped TiO_2, and sulfur-doped TiO_2 containing other metals

TiO_2 particles having an anatase phase and specific surface area of 70 m^2/g were obtained from FUJICO CO., Ltd. Sulfur-doped TiO_2 having an anatase phase and a specific surface area of 50 m^2/g were also obtained from FUJICO CO., Ltd. as were the metals used as additives: copper, silver, and nickel. Sulfur-doped TiO_2 production refers to method of Ohno *et al.*[24].

Photocatalyst thin films were prepared by a High Velocity Oxygen Fuel system (FUJICO Co., Ltd.)[15]. Photocatalysts were sprayed on an anodized aluminum board (FUJICO CO., Ltd.). In the case of TiO_2 or sulfur-doped TiO_2, 0.07 g of each additive-free photocatalyst were sprayed on 25 cm^2 (a square with both sides 5 cm) of anodized aluminum board using the following conditions. The spraying distance was 100–150 mm. The spray rate was 500–1000 mm/sec. In addition, the flame temperature was approximately 1,500°C with the particle temperature not exceeding 400°C. The photocatalystic film thickness was about 100 μm. In the case of sulfur-doped TiO_2 containing the metals, the metals mixed sulfur-doped TiO_2 using ball milling. Well-blended mixture was treated as discussed above. The chemical composition of the sprayed board was determined by X-ray fluorescence (RIX3001, Rigaku Co., Ltd.) and X-ray diffraction (RINT2500V, Rigaku Co., Ltd.).

2.2. Bacterial strains and culture

Staphylococcus aureus IFO 12732 was obtained from the Institute for Fermentation, Osaka, Japan. A stock of *S. aureus* was maintained on a Nutrient Agar (NA, Nissui Pharamaceutical Co. Ltd.) slant. The bacterium from the stock culture was pre-cultured at 30°C for 2 days on a fresh NA plate and used for estimating bactericidal activity.

S. aureus cultures were grown in 100 mL of Nutrient Broth (Nissui Pharamaceutical Co., Ltd.) in 300 mL flasks that had been inoculated from slants, and incubated at 30°C on a rotary shaker. After 24 h, the broth was centrifuged at $4,000 \times g$ for 20 min and the cell pellet was re-suspended in sterile water.

2.3. Bactericidal activity of the photocatalytic reaction

Before performing the experiment, the photocatalyst-coated substrate was washed with acetone and was then illuminated with UV light (GL-15, Hitachi Ltd.) for 12 h to remove contamination. The *S. aureus* suspension was adjusted to approximately 10^6 CFU/mL with sterilized water and 30 mL were poured into glass petri dishes (90 mm diameter). The board coating with photocatalyst was put on the petri dish. Petri dishes containing substances were then placed under an incandescent lamp (FDL27Ex-N, National Co., Ltd.) to initiate the photocatalytic reaction. To control illumination intensity, the distance between the light source and photocatalyst-coated substrate was adjusted. For example, a distance of approximately 30 cm was equivalent to 1700 lx as measured using a light meter (LM-331, ASONE Co., Ltd). The basic irradiation dose was 1700 lx which was varied for kinetic analysis by extending the duration of illumination. Unless specified, illumination were carried out at 30°C. After illumination, 1 mL of the *S. aureus* solutions was sampled to estimate bactericidal activity by quantifying survival. Colonies were counted at least 48 h after plating on NA. The results were the average values of three experiments.

3. Results

3.1. Bactericidal activities of TiO_2 versus sulfur-doped TiO_2 under visible-light irradiation

Fig. 1 shows survival of *S. aureus* on TiO_2 and sulfur-doped TiO_2 substrates as a function of time. As shown in Fig. 1A, no obvious changes in survival were observed in the absence of visible-light illumination. When the TiO_2-coated substrate was stored under visible-light, survival of *S. aureus* decreased hardly. In contrast, survival of *S. aureus* decreased to a far greater extent on the sulfur-doped TiO_2.

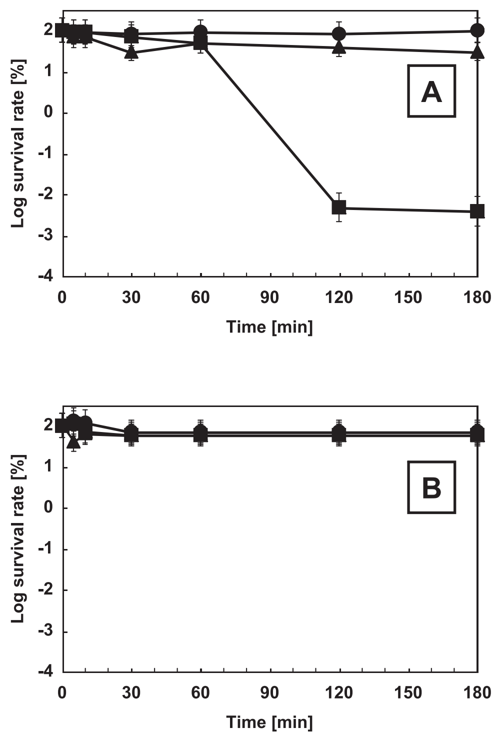

Fig. 1. Changes in survival of *S. aureus* versus illumination time. The bacterial suspension (10^6 CFU/mL) was incubated under (A) visible-light illumination (1700 lx) or (B) in the dark. Symbols represent: —●—, blank; —▲—, TiO_2; and —■—, Sulfur doped TiO_2.

Fig. 1B shows that all substrates were not toxic for *S. aureus* under dark conditions.

3.2. Effect of combinations of various metals and sulfur-doped TiO_2 on bactericidal activity under visible-light irradiation

Fig. 2 shows survival of *S. aureus* on sulfur-doped TiO_2 containing copper, silver, and nickel as a function of time. The metals were added at a ratio of 10% (w/w). As shown in Fig. 2A, a large decrease in the number of viable cells was observed on the illuminated copper-containing sulfur-doped TiO_2 film, demonstrating enhanced photo-killing activity. Viable cells of *S. aureus* were undetected levels within only ~10 min under visible-light irradiation. In the case of silver supplementation, almost no surviving cells were detected after 120 min of irradiation. Nickel-supplemented sulfur-doped TiO_2, had somewhat less killing activity as some surviving cells were still present after 180 min of irradiation. Overall however, bactericidal activity of sulfur-doped TiO_2 supplemented with the metals was higher than sulfur-doped TiO_2 alone. No obvious changes in survival were observed upon exposure of cells to the various substrates in the dark (Fig. 2B).

3.3. Effect of amount of copper in sulfur-doped TiO_2 on bactericidal activity under visible light

The effect of copper concentration (1–20% [w/w]) in sulfur-doped TiO_2 on bactericidal activity under visible-light irradiation was tested. As shown in Fig. 3A, survival of *S. aureus* decreased significantly in the presence of 4 to 10% copper, with complete killing of *S. aureus* observed within only ~10 min. Sulfur-doped TiO_2 containing 12–20% (w/w) copper also had greater antibacterial activity against *S. aureus* than sulfur-doped TiO_2 alone. In this case, killing of *S. aureus* was complete within ~120 min under visible light. Addition of 1–3% (w/w) copper was found to enhance the bactericidal activity of sulfur-doped TiO_2 to a lesser extent than higher additions. In fact, the bactericidal activity of sulfur-doped TiO_2 containing 1% (w/w) copper was found to be equivalent to that without copper. As shown in Fig. 3B, survival of *S. aureus* on sulfur-doped TiO_2 substrate containing various amounts of added copper was fairly constant under dark room.

3.4. Utility of copper-containing sulfur-doped TiO_2 under visible light

In order to examine the utility of copper-containing (5%) sulfur-doped TiO_2 using visible-light irradiation, we assessed survival of *S. aureus* under different illumination intensities covering a range typical of work and home environments (250–1700 lx). According to Toguh[30], 2000 lx of illumination is equivalent to exposure at a north window, 750 lx is equivalent to an office desktop, and 200 lx is equivalent to a drawing room. As shown in Fig. 4, survival of *S. aureus*

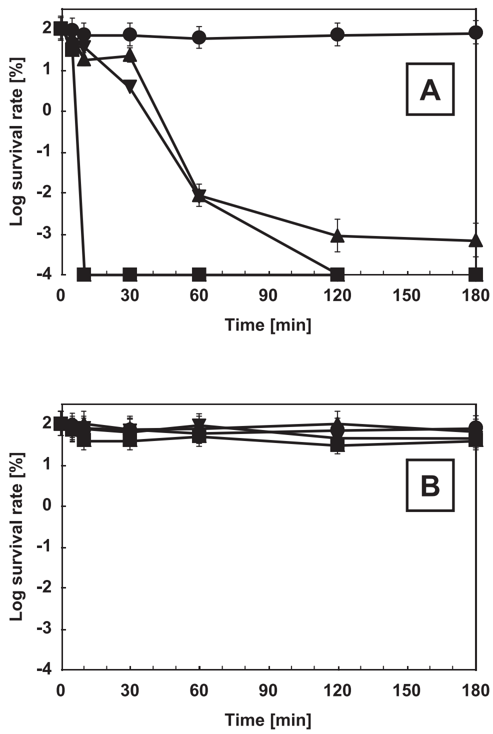

Fig. 2. Bactericidal activity of metal-containing sulfur-doped TiO_2. The bacterial suspension (10^6 CFU/mL) was incubated under (A) visible-light illumination (1700 lx) or (B) in the dark. Symbols represent: —●—, blank; —■—, copper-containing sulfur-doped TiO_2; —▼—, silver-containing sulfur-doped TiO_2; and —▲—, nickel-containing sulfur-doped TiO_2.

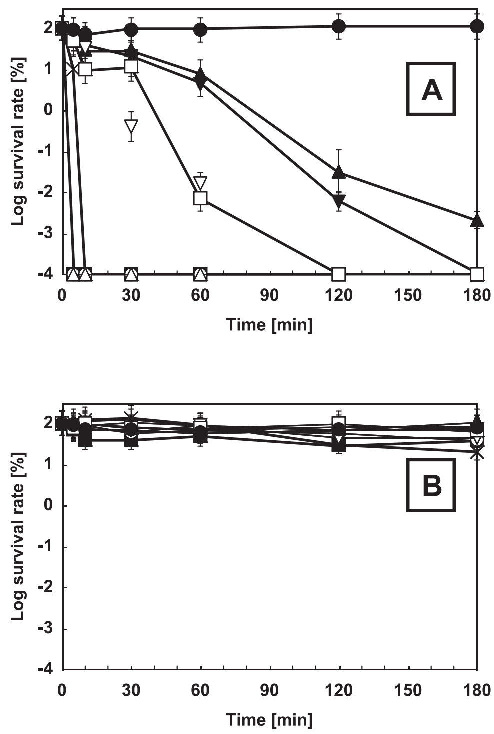

Fig. 3. Influence of the amount of copper in sulfur-doped TiO_2 on bactericidal activity. The bacterial suspension (10^6 CFU/mL) was incubated under (A) visible-light illumination (1700 lx) or (B) in the dark. Symbols represent: —●—, blank(Non-photocatalyst); —▲—, 1%; —▼—, 3%; —■—, 4%; —×—, 5%; —○—, 7%; —△—, 10%; —▽—, 12%; and —□—, 20%.

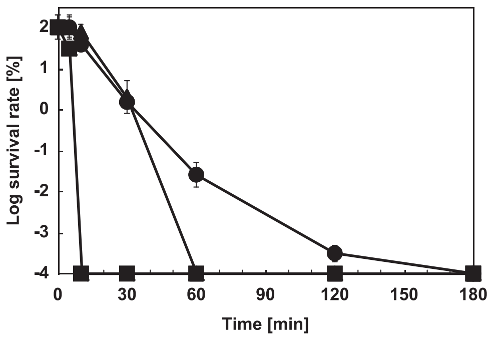

Fig. 4. Influence of illumination intensity on bactericidal activity of 5% copper-containing sulfur-doped TiO_2 under visible-light illumination. Symbols represent: —●—, 250 lx; —▲—, 650 lx; and —■—, 1700 lx.

decreased dramatically on copper-containing sulfur-doped TiO_2 irradiated with approximately 1700 lx. Killing of *S. aureus* was complete within only ~10 min under visible-light irradiation. At approximately 650 lx, almost no *S. aureus* cells survived after 60 min of irradiation. When the copper-containing sulfur-doped TiO_2 was irradiated with 250 lx, *S. aureus* was completely killed after 180 min. The greater the illumination intensity, the greater the bactericidal activity.

4. Discussion

The sulfur-doped TiO_2 showed high antibiotic activity than non-doped TiO_2 under visible-light irradiation (Fig. 1). This *S. aureus* inactivation was caused by photocatalytic reaction because the sulfur-doped TiO_2 did not show the antibiotic activity under dark room. It should be emphasized that the *S. aureus* survival curve on sulfur-doped TiO_2 under visible-light did not follow a simple single exponential decay process as a function of illumination time. After 120 min of visible-light irradiation, survival of *S. aureus* barely decreased. However, the *S. aureus* inactivation was not reached within the experimental time completely.

Silver, copper, and nickel have been reported to possess antibacterial activity against *S. aureus*[21)]. We choose these metals because of their relatively low toxicity towards human cells[2,12,34)] and because silver and copper in particular have been studied extensively due to their high antibacterial activity[5,12,21-23,31)]. The antibacterial activity of these metals has been compared and in decreasing order of activity, has been ranked: silver, copper, and nickel[12,21,23)]. In order to enhance bactericidal activity in the dark and under very weak UV illumination, (*i.e.*, during exposure to indoor UV light), TiO_2 films containing copper and silver have been developed[29,33)]. However, in the present study, the greatest enhancement of antibacterial activity was observed upon addition of copper in the presence of visible light. As shown in Fig. 2B, survival of *S. aureus* was virtually unchanged on sulfur-doped TiO_2 substrate containing copper, silver, or nickel in the absence of visible light. This result suggested the antibiotic activities of sulfur-doped TiO_2 containing some metals were not caused by chemical toxicity of additive metals under visible-light irradiation. Photo-catalytic activity and associated acetaldehyde levels of metal-ion modified TiO_2 was reported to be greater than unmodified TiO_2 upon irradiation with both ultraviolet and visible-light[22)]. According to this report, the effect of copper addition was greater than that of nickel. It was suggested that the antibacterial activity of the sulfur-doped TiO_2 containing metal was induced by photo-catalysis.

The antibiotic activity of sulfur-doped TiO_2 containing copper increased with increase in copper. Sulfur-doped TiO_2 containing copper which the amount was 4–10% (w/w) showed the highest of antibiotic activity. Above the amount of 10%, the antibiotic activity of sulfur-doped TiO_2 containing copper gradually decreased with increase in copper because the copper was thought to cover active site. Fujihara *et al.*[10)] suggested the large excess amount of Fe (III) ions on TiO_2 was thought to function as a recombination center between electrons and holes. In the case of copper, it is possible to cause similar phenomenon. Moreover, copper ions interacting with the cytoplasmic membrane interfered with both membrane structure and function, leading to loss of metabolic activity[31)]. Sunada *et al.*[29)] speculated that weak UV irradiation of copper-containing TiO_2 initially resulted in partial decomposition of the outer membrane of the cell envelope by a photo-catalytic process. This in turn was followed by uptake of copper ions into the cytoplasmic membrane due to copper ion-mediated disruption of membrane structure. The bactericidal mechanism of copper-containing sulfur-doped TiO_2 was thought to be similar to that of copper-containing TiO_2. Thus, the amount of copper in the sulfur-doped TiO_2 may be one of most important factors controlling photo-catalytic bactericidal activity. Further studies are needed to determine precisely how copper enhances bactericidal activity of sulfur-doped TiO_2.

Exposure of humans to UV light at the necessary level, however, would cause significant damage to the skin and eyes[14,28)], thus limiting the potential for use of TiO_2 substrates in environments where humans would be exposed. Public environments are ideal places for transmission of pathogens[32)]. The visible light-induced bactericidal activity of copper-containing sulfur-doped TiO_2 offers the potential for use as a disinfectant in public areas, specifically indoor environments without adequate air circulation, such as public toilets, schools, hospitals, airports, hotels, and subways. A method able to continuously disinfect surfaces of door handles and push buttons, for example, could potentially limit the spread of pathogens[4)]. Chlorination is often used for disinfection because it is inexpensive and convenient. However, among its drawbacks are trihalomethanes generated as by-products of its reaction with organic matter, phytotoxicity, and an unpleasant taste when used in drinking water[8)]. The present study demonstrated that copper-containing sulfur-doped TiO_2 has better visible light-catalyzed bactericidal activity against *S. aureus*, a significant human pathogen, than TiO_2 alone or sulfur-doped TiO_2. The sulfur-doped TiO_2 under visible-light irradiation have the potential to overtake chlorine sterilization. These results suggest that this material has the potential for use in environmental applications.

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