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Rutile-TiO_2/PtO_2 Glass Coatings Disinfects Aquatic *Legionella pneumophila* via Morphology Change and Endotoxin Degradation under LED Irradiation

Ryosuke Matsuura [1], Arisa Kawamura [1], Yasunobu Matsumoto [1,2], Takashi Fukushima [3], Kazuhiro Fujimoto [3], Heihachiro Ochiai [3], Junichi Somei [3] and Yoko Aida [1,2,*]

[1] Laboratory of Global Infectious Diseases Control Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan; matsuura-ryosuke@g.ecc.u-tokyo.ac.jp (R.M.); arisa-k@g.ecc.u-tokyo.ac.jp (A.K.); aymat@g.ecc.u-tokyo.ac.jp (Y.M.)
[2] Laboratory of Global Animal Resource Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
[3] Kaltech Co., Ltd., 3-3-7 Bakuromachi, Chuo-ku, Osaka 541-0059, Japan; fukushima.takashi@kaltec.co.jp (T.F.); fujimoto.kazuhiro@kaltec.co.jp (K.F.); ochiai.heihachiro@kaltec.co.jp (H.O.); somei.junichi@kaltec.co.jp (J.S.)
* Correspondence: yoko-aida@g.ecc.u-tokyo.ac.jp

Abstract: *Legionella pneumophila* (*L. pneumophila*) is the causative agent of Legionnaires' disease and Pontiac fever, collectively known as legionellosis. *L. pneumophila* infection occurs through inhalation of contaminated aerosols from water systems in workplaces and institutions. The development of disinfectants that can eliminate *L. pneumophila* in such water systems without evacuating people is needed to prevent the spread of *L. pneumophila*. Photocatalysts are attractive disinfectants that do not harm human health. In particular, the TiO_2 photocatalyst kills *L. pneumophila* under various conditions, but its mode of action is unknown. Here, we confirmed the high performance of TiO_2 photocatalyst containing PtO_2 via the degradation of methylene blue (half-value period: 19.2 min) and bactericidal activity against *Escherichia coli* (half-value period: 15.1 min) in water. Using transmission electron microscopy, we demonstrate that the disinfection of *L. pneumophila* (half-value period: 6.7 min) by TiO_2 photocatalyst in water is accompanied by remarkable cellular membrane and internal damage to *L. pneumophila*. Assays with limulus amebocyte lysate and silver staining showed the release of endotoxin from *L. pneumophila* due to membrane damage and photocatalytic degradation of this endotoxin. This is the first study to demonstrate the disinfection mechanisms of TiO_2 photocatalyst, namely, via morphological changes and membrane damage of *L. pneumophila*. Our results suggest that TiO_2 photocatalyst might be effective in controlling the spread of *L. pneumophila*.

Keywords: TiO_2/PtO_2-coated glass; TiO_2/PtO_2-coated sheet; rutile TiO_2 photocatalyst modified with PtO_2; *Legionella pneumophila*; disinfection; morphology change; endotoxin degradation; aquatic environment; LED irradiation

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1. Introduction

Since the 1970s, many kinds of emerging infectious diseases, such as acquired immunodeficiency syndrome (AIDS), enterohaemorrhagic *Escherichia coli* infection, Ebola virus disease, and coronavirus disease 2019 (COVID-19), have been reported, which has led to an increasing interest in public health measures to control their spread. Various methods for disinfection including drugs, antibiotics, and sterilization using light (e.g., ultraviolet [UV]) have been actively researched. Photocatalysts are attractive choices as disinfectants because they do not harm human health. For example, although the International Agency for Research on Cancer classified TiO_2 as possibly carcinogenic to humans (group 2B), it noted that there was limited evidence of carcinogenicity in experimental animals, and two large epidemiological studies of workers employed in the TiO_2 production industry in the United

States and Europe clearly demonstrated that no increased risks were found for lung cancer, total cancers, or other causes of death [1,2]. In addition, porous TiO_2 synthesized from Ti and ammonium hydrogen carbonate (NH_4HCO_3) was found to have no cytotoxic effects on human HeLa and Vero cells [3]. Irradiation with light activates the photocatalytic reaction of TiO_2, the most common photocatalyst, generating reactive oxygen species (ROS), such as hydroxyl (·OH) and superoxide radicals ($O_2\cdot^-$) on the surface of the TiO_2 [4]. The band gap energy and peak photoexcitation wavelength of rutile-type TiO_2 have been determined to be 3.05 eV and 406.5 nm, respectively [5]. Rutile-type TiO_2 can be excited by a 405 nm LED, which is an inexpensive light source and harmless compared to UVC [6]. ROS have strong oxidizing power and mineralize organic compounds, and this mineralization leads to the degradation of membranes, proteins, and genetic material, killing microorganisms and viruses [4]. Indeed, TiO_2 photocatalysts can kill various microorganisms [4], including *Bacillus cereus* [7,8], *Escherichia coli* (*E. coli*) [9,10], *Enterobacter spp.* [11,12], *Porphyromonas gingivalis* [13], *Salmonella spp.* [7,11,14–16], and *Vibrio parahaemolyticus* [14,17]. TiO_2 photocatalysts can decompose the *E. coli* membrane during the process of sterilization [9,10]. TiO_2 photocatalysts can also degrade *E. coli* endotoxin [18,19], which is a component of the outer membrane of Gram-negative bacteria and a causative agent of sepsis. Therefore, killing the microorganism and degrading endotoxin are both important functions of photocatalysts. In the previous studies, various types of TiO_2, such as TiO_2 synthesized from $TiCl_4$, HCl, $2(NH_4)HCO_3$, and H_2O_2 [7]; commercial TiO_2 (P-25, Degussa–Huels AG, Frankfurt am Main, Germany; Yakuri Pure Chemicals Co., Osaka, Japan) [8,11,14,15]; Ag core and TiO_2 shelled nanoparticles [9,10]; TiO_2 mixed with 5-, 10-, 15-, 20-tetraphenyl-21H, 23H-porphine nickel [12]; TiO_2 made from a titanium peroxo complex [13]; and TiO_2-coated glass by plasma-enhanced chemical vapor deposition [16] were used. Moreover, in those studies, TiO_2 was coated on various materials, such as quartz, silver, glass, and stainless-steel wires [7,9,10,12,13,16]. This suggests that TiO_2 photocatalysts might have broad applicability in many different settings.

Legionellosis, which includes Legionnaires' disease and Pontiac fever, is an emerging infectious disease. *Legionella pneumophila* (*L. pneumophila*), the causative agent of legionellosis, is a Gram-negative, aerobic, rod-shaped bacteria. *Legionella spp.* was first reported in 1976 when many people attending an American Legion event succumbed to legionellosis [18]. The following year, *L. pneumophila* was recognized as the major causative agent of legionellosis [19]. *L. pneumophila* damages human health, and the fatality rate of Legionnaires' disease reported by the Centers for Disease Control and Prevention is approximately 9% [20]. The incidence of Legionella infection has increased in both the United States and Europe [20,21]. Infection of *L. pneumophila* occurs through inhalation of contaminated aerosols, and *L. pneumophila* infects alveolar macrophages [22]. *L. pneumophila* is naturally ubiquitous in aquatic and damp environments such as lakes, rivers, composted materials, and moist soil as intracellular bacteria living inside eukaryotes such as amebae [21]. In addition, many sources of *L. pneumophila* have been reported around workplaces [23] and in potable water [24,25], construction area sinks [26], showerheads [27], car air conditioners [28], humidifiers [29], dental unit waterlines [30], and especially hot springs [31–35]. Human–human transmission of *L. pneumophila* is non-existent or very rare [20,36]. There has been much interest in the development of disinfectants that can sterilize water from workplaces without evacuating people in order to prevent the spread of *L. pneumophila*, with various disinfectants under consideration.

It was previously reported that a TiO_2 photocatalyst can kill *L. pneumophila* [37–40]. Indeed, *L. pneumophila* in aerosols [37], under laminar flow [38], under semi-dry conditions [39], and in rainwater [40] have been killed by the TiO_2 photocatalyst. These previous reports suggest that the TiO_2 photocatalyst has potentially widespread applications in the control of *L. pneumophila*. However, damage to the membrane and endotoxin of *L. pneumophila* by the TiO_2 photocatalyst has not yet been reported. Moreover, the mechanisms by which the TiO_2 photocatalyst kills *L. pneumophila* remain unknown.

To investigate whether rutile-type TiO_2/PtO_2 (hereafter "TiO_2") -coated glass exerts photocatalytic activity in water by excitation of light with a wavelength of 405 nm, we first evaluated the performance of TiO_2-coated glass in water by degradation of methylene blue and killing of *E. coli*. Furthermore, we demonstrated the susceptibility to disinfection in water of *L. pneumophila*, and then clarified the disinfection mechanism of TiO_2 by observing the morphology of *L. pneumophila* using transmission electron microscopy (TEM). Finally, we performed the limulus amebocyte lysate (LAL) assay [41,42] and silver staining to detect the degradation of the *L. pneumophila* endotoxin.

2. Results

2.1. Photocatalytic Degradation of Methylene Blue in Water by TiO_2 Photocatalyst

To clarify whether TiO_2-coated glass (5 cm × 5 cm) exerts photocatalytic activity in water by excitation of light with a wavelength of 405 nm, we first tested the decomposition of methylene blue in water purified by ion-exchange. As shown in Figure 1A, TiO_2-coated glass decomposed methylene blue by excitation of light in a time-dependent manner. The half-value period was 19.2 min (R^2 = 0.9915) and the curve flattened between 90 and 120 min after the light emitting diode (LED)-TiO_2 photocatalytic reaction (Figure 1B). In addition, reaction rate was estimated as followed equation:

$$r\ (\mathrm{nM/min}) = 0.107\ [methlene\ blue]^{1.06} \quad (1)$$

where r is the reaction rate; 0.107 is reaction rate constant; [*methylene blue*] is the concetration of methylene blue; and 1.06 is the order of reaction. This equation suggested that this degradation reaction was first order reaction. By contrast, neither TiO_2-coated glass under dark conditions (TiO_2 + Dark) nor glass alone under dark conditions (Glass + Dark) decreased absorbance. As expected, glass under the light condition (Glass + Light) decreased absorbance because it is known that methylene blue is photodegradable. However, degradation of methylene blue by TiO_2 under light conditions (TiO_2 + Light) was more efficient than that by the glass under light conditions. These results demonstrate that the photocatalytic activity of TiO_2-coated glass degrades methylene blue in water.

2.2. Disinfection of E. coli in Water by TiO_2 Photocatalyst

The TiO_2 photocatalyst can kill many kinds of microorganisms [4]. Therefore, we examined the bactericidal effect of TiO_2-coated glass in water using *E. coli*, the most studied lab bacterium. As shown in Figure 2A, TiO_2-coated glass was placed in a 10-cm diameter dish on the shaker, 30 mL of phosphate-buffered saline (PBS) containing *E. coli* with a titer of 1×10^8 colony forming unit (CFU)/mL was added, and the dish was then exposed to LED light (placed above the dish) with a wavelength of 405 nm. The *E. coli* was killed such that its titer reached below the detection limit in a time-dependent manner within 8 h (Figure 2B). In addition, the Glass + Light group also killed *E. coli* in a time-dependent manner. This is because light with a wavelength of 405 nm has a bactericidal effect. Notably, the disinfection effect for *E. coli* in the TiO_2 + Light group was stronger than that in the Glass + Light group (Figure 2C,D), and the titer was significantly ($p < 0.05$) lower at 6 h after light excitation (Figure 2C). In addition, the half-value periods of the TiO_2 + Light group and Glass + Light group were 15.1 min (R^2 = 0.97), and 25.8 min (R^2 = 0.95), respectively, suggesting that the TiO_2 + Light group disinfects *E. coli* 1.71 times faster than the Glass + Light group. These results suggested that TiO_2-coated glass has a bactericidal effect in water and that the photocatalytic reaction can kill *E. coli*.

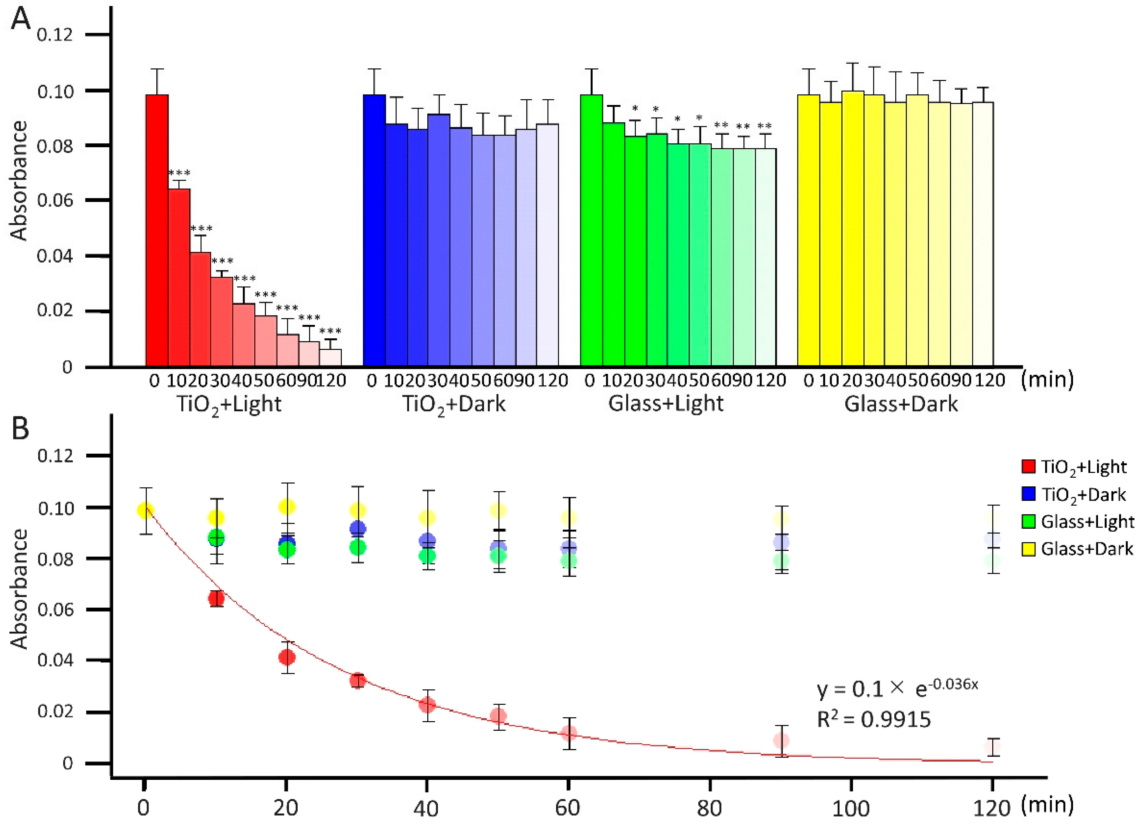

Figure 1. Photocatalytic degradation of methylene blue in water. In the "TiO_2 + LED light" group, TiO_2-coated glass in 100 mL of 12.5 nM methylene blue in water purified by ion-exchange was irradiated with a LED with a wavelength of 405 nm; absorbance was calculated at 660 nm from 0 to 120 min. As a control, methylene blue was incubated with either TiO_2-coated glass without LED light ("TiO_2 + Dark" group) or both glass and LED light ("Glass + Light" group), or glass without LED light ("Glass + Dark" group). (**A**) Each column and error bar represent the mean ± standard deviation (SD) for three experiments. All values in each group were compared with the 0 min sample by two-way analysis of variance (ANOVA) with Dunnett's test. The asterisk indicates the statistical difference (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). (**B**) Exponential regression analysis between absorbance of methylene blue and irradiation time from 0 to 60 min before flattening out. R^2 indicates the coefficient of determination.

2.3. Disinfection of *L. pneumophila* in Water by TiO_2 Photocatalyst

L. pneumophila grows in aquatic environments and can cause serious health problems in humans [20,21]. Therefore, it is essential to confirm sterilization ability of TiO_2-coated glass against *L. pneumophila* in water. Thus, 30 mL of solution including *L. pneumophila* with a titer of 1×10^7 CFU/mL and TiO_2-coated glass was irradiated by an excitation light from 0 to 8 h. As shown in Figure 3A,C, the titer of *L. pneumophila* drastically decreased in a time-dependent manner for the TiO_2-coated glass with excitation light and reached undetectable levels at 4 h. Although the Glass + Light group also decreased the titer of *L. pneumophila*, the TiO_2 + Light group showed a stronger bactericidal effect, and the titer was significantly lower at 1 h ($p < 0.05$) and 2 h ($p < 0.001$) (Figure 3B). In addition, the half-value periods of the TiO_2 + Light group and the Glass + Light group were 6.7 min ($R^2 = 0.99$) and 12.0 min ($R^2 = 0.99$), respectively, suggesting that the TiO_2 + Light group disinfected water with *L. pneumophila* 1.80 times faster when compared with the Glass + Light group. These results show that TiO_2-coated glass can sterilize water with both *E. coli* and *L. pneumophila*, suggesting that TiO_2-coated glass might be an effective sterilizing agent for all Gram-negative bacteria.

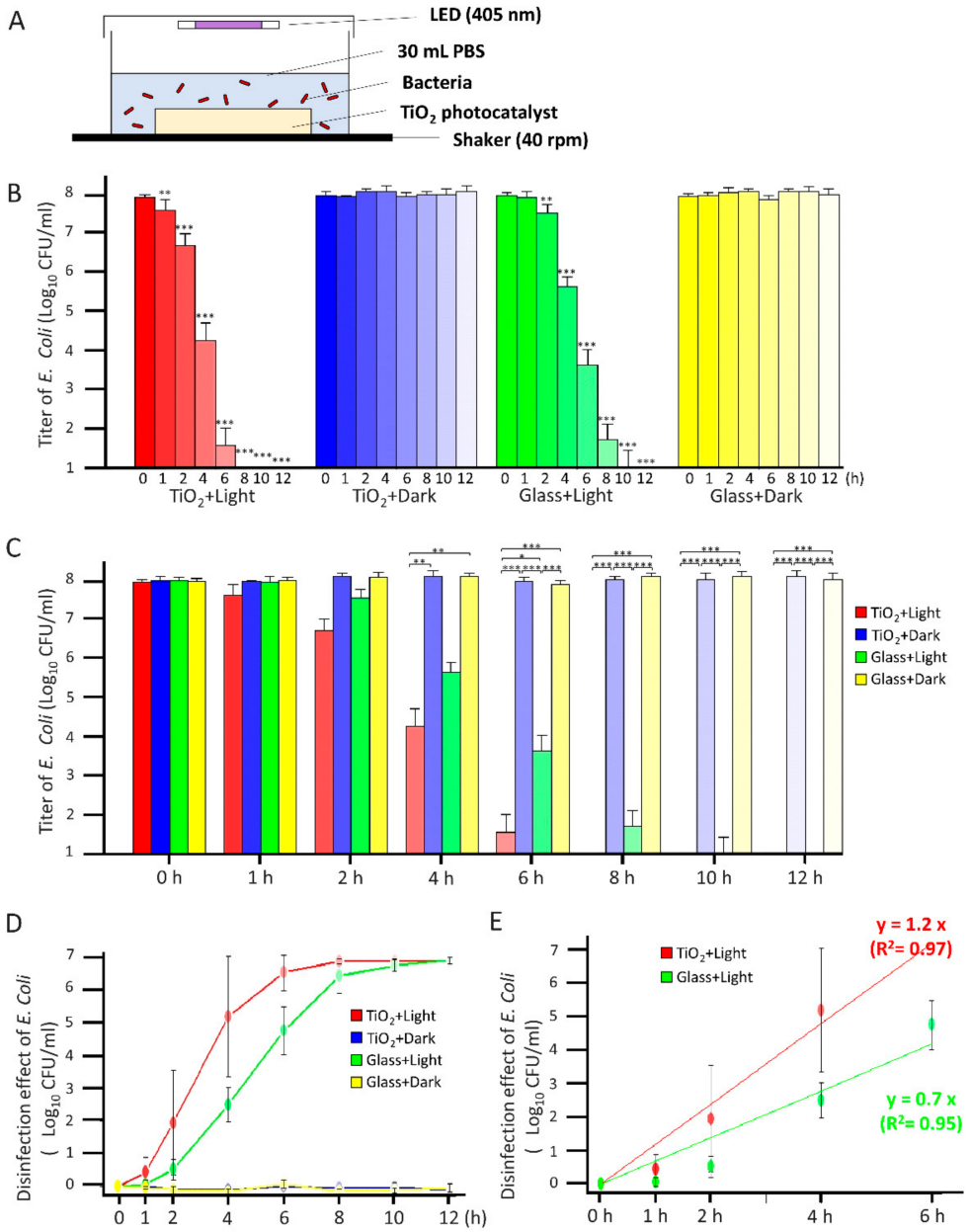

Figure 2. Disinfection of *E. coli* by TiO_2 photocatalyst. (**A**) Schematic diagram of disinfection of bacteria by the TiO_2 photocatalyst. (**B**) In the "TiO_2 + Light" group, 30 mL of *E. coli* with a titer of 1×10^8 CFU/mL was added to a 10-cm diameter dish with TiO_2-coated glass; then, 1 mL samples were taken at the marked hourly intervals. As a control, *E. coli* was incubated with TiO_2-coated glass without LED light ("TiO_2 + Dark" group), glass and LED light ("Glass + Light" group), or glass without LED light ("Glass + Dark" group). Each column and error bar represent the mean ± standard deviation (SD) of three experiments. Significance between 0 min and other time points in each group was determined using two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. (**C**) Significance between each condition in each time point was determined using two-way ANOVA followed by Tukey's multiple comparisons test. Asterisks indicate statistical difference (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). (**D**) The line graph shows the time-dependent changes in disinfection effects of *E. coli*. Decrement of titer of *E. coli* calculated according to the following equation: Decrement of titer of *E. coli* = Titer of *E. coli* at each time point − Titer of *E. coli* at 0 h. (**E**) Linear regression analysis between disinfection effect of *E. coli* and irradiation time before levelling out. R^2 indicates the coefficient of determination.

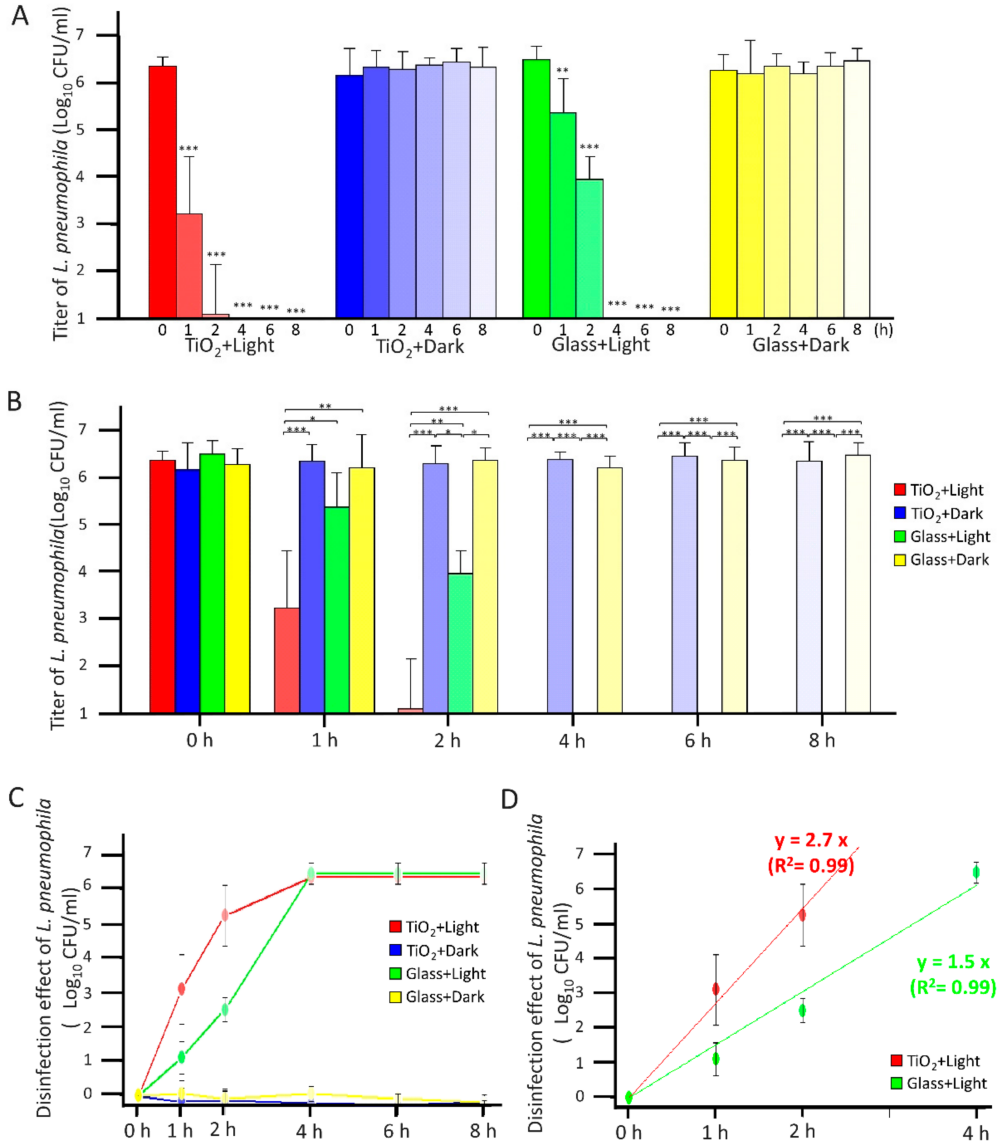

Figure 3. Disinfection of *L. pneumophila* by the TiO_2 photocatalyst. (**A**) In the "TiO_2 + Light" group, 30 mL of *L. pneumophila* with a titer of 1×10^7 CFU/mL was added to a 10-cm diameter dish with TiO_2-coated glass and exposed to light from a LED with a wavelength of 405 nm. Samples (1 mL) were taken at the indicated intervals. During irradiation, the dish was shaken at 40 rpm. As a control, *L. pneumophila* was incubated with either TiO_2-coated glass without LED light ("TiO_2 + Dark" group), glass and LED light ("Glass + Light" group), or glass without LED light ("Glass + Dark" group). Significance between 0 min and other time points in each group was determined using two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. Asterisks indicate statistical difference (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). (**B**) Significance between each condition in each time point determined using two-way ANOVA followed by Tukey's multiple comparisons test. Asterisks indicate statistical difference (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). (**C**) The line graph shows the time-dependent changes in disinfection effects of *L. pneumophila*. Decrement of titer of *L. pneumophila* calculated according to the following equation: Decrement of titer of *L. pneumophila* = Titer of *L. pneumophila* at each time point − Titer of *L. pneumophila* at 0 h. (**D**) Linear regression analysis between disinfection effect of *L. pneumophila* and irradiation time before levelling out. R^2 indicates the coefficient of determination.

2.4. Morphological Changes in L. pneumophila Induced by TiO_2 Photocatalytic Disinfection

It has been previously reported that photocatalytic reactions disrupt the bacterial cell membrane [9,10]. To clarify the mechanism of *L. pneumophila* disinfection by a photocatalytic reaction, we observed the morphology of *L. pneumophila* using TEM. *L. pneumophila* with a titer of 2.91×10^7 CFU/mL was irradiated by excitation light with TiO_2-coated glass for 24 h; TEM images of *L. pneumophila* before and after the photocatalytic reaction were obtained (Figure 4). Under this condition, 99.6% of *L. pneumophila* in the TEM image was killed (Figure 4E). As shown in Figure 4A (left panel) and Figure 4B (left panel), untreated *L. pneumophila* before the photocatalytic reaction had a normal membrane, and 70.5% of the cells were stained black with uranyl acetate (Figure 4D). By contrast, after 24 h of the photocatalytic reaction, membranes had broken down (lacking the fluffy edge) and cells were not stained, as shown in Figure 4A (right panel) and Figure 4B (right panel). These results suggest that disinfection by photocatalytic reaction is based on significant damage to cell membranes and interiors. Thus, after 24 h of the photocatalytic reaction, 80.2% of cells in low magnification TEM images were dead *L. pneumophila* (shown by the blue arrows in Figure 4C, right panel). The proportion of dead *L. pneumophila* was significantly higher ($p < 0.001$) in the treatment group compared with that in the non-treatment group (Figure 4D). These results suggest that damage to membranes is one mechanism for the photocatalytic sterilization of *L. pneumophila* in water.

2.5. L. pneumophila Endotoxin Degradation by TiO_2 Photocatalyst

Endotoxin is a structural component of the outer membrane of Gram-negative bacteria and is a pyrogenic substance [43,44]. Since the TiO_2 photocatalytic reaction damaged the membrane of *L. pneumophila*, it was considered likely that endotoxin would also be released. To test for release of endotoxin in water, 30 mL of *L. pneumophila* with a titer of 10^7 CFU/mL was irradiated with excitation light and TiO_2-coated glass from 0 to 12 h, and the concentration of endotoxin in the water was measured. Interestingly, in the TiO_2 + Light group, the concentration of endotoxin increased in a time-dependent manner (Figure 5A). This corroborates our TEM results showing the morphological changes in *L. pneumophila* by TiO_2 photocatalytic disinfection. Likewise, in the Glass + Light group, the concentration of endotoxin also increased in a time-dependent manner. These increases in endotoxin concentration resemble those seen when Gram-negative bacteria are treated with drugs such as antibiotics [43]. However, the increase in endotoxin concentration in the TiO_2 + Light group was limited compared with that in the Glass + Light group. This might be because of endotoxin degradation by the photocatalytic reaction but not by LED light.

To determine whether the TiO_2 photocatalyst induces the degradation of endotoxin from *L. pneumophila* in water, we extracted endotoxin from *L. pneumophila*, dropped samples onto the TiO_2-coated glass sheet (1 cm × 1cm) and irradiated the sheets with 405 nm light for 24 h (Figure 5B). Then, a sample was collected by washing with 10 mM Tris-HCl (pH = 8.0), and the concentration was measured using the LAL method. As shown in Figure 5C, the concentration of endotoxin was significantly decreased (by 81%; $p < 0.05$) during the 24 h photocatalytic reaction (Figure 5C). Untreated endotoxin extracted from *L. pneumophila* was detected by silver staining as any band between 15 and 20 kDa; however, these bands disappeared after the 24 h photocatalytic reaction (Figure 5D). Moreover, band density of endotoxin was significantly decreased (by 78%; $p < 0.05$) in the treated sample after 24 h photocatalytic reaction as compared to that in the sample at 0 h (Figure 5E). These results suggest that the TiO_2 photocatalyst degrades endotoxin released from *L. pneumophila*.

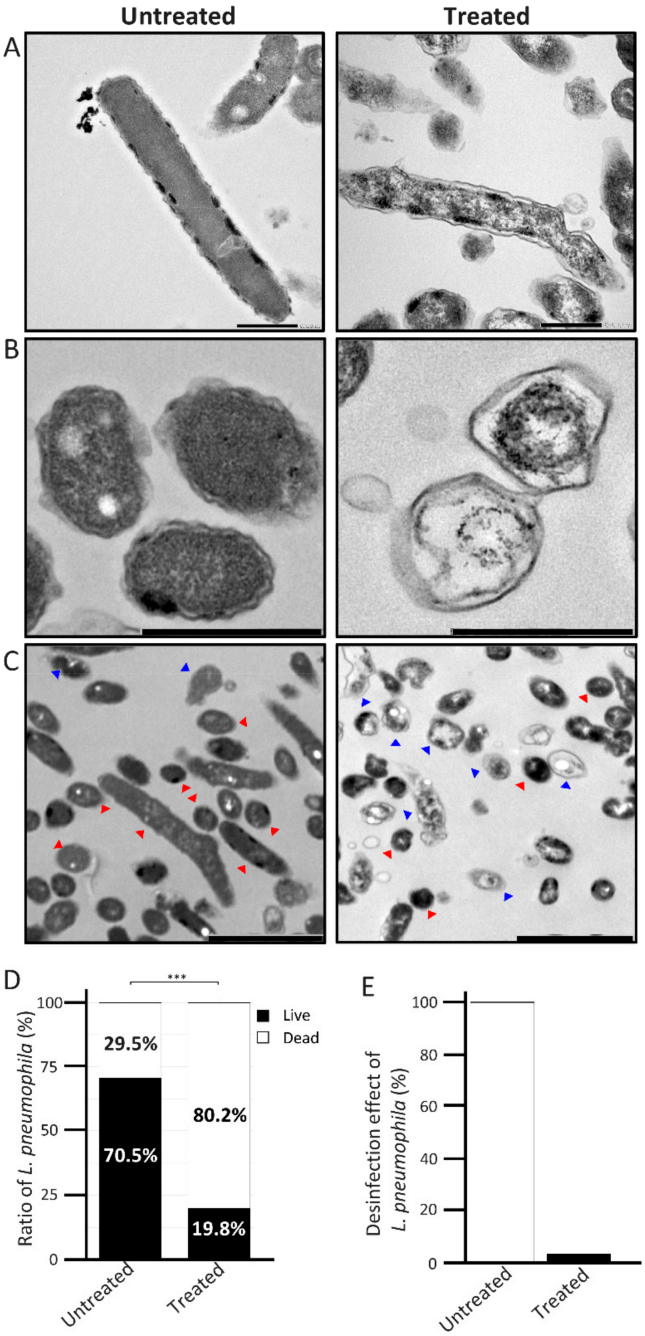

Figure 4. Morphological changes in *L. pneumophila* owing to TiO_2 photocatalytic disinfection. A 30 mL sample of *L. pneumophila* with a titer of 1×10^8 CFU/mL was added to a 10-cm diameter dish with TiO_2-coated glass and exposed to a LED with a wavelength of 405 nm for 0 or 24 h. The sample treated by photocatalytic disinfection for 24 h (Treated) and the untreated sample (Untreated) were fixed with 2.5% glutaraldehyde for transmission electron microscopy (TEM) negative staining. TEM images showing the sagittal plane (**A**) and transverse plane (**B**) of *L. pneumophila*. Bar = 500 nm. (**C**) Low magnification TEM images for counting living and dead *L. pneumophila*. Red arrows show living *L. pneumophila,* and blue arrows show dead *L. pneumophila*. Bar = 2.0 µm. (**D**) Dead and live *L. pneumophila* were counted by eye from three low magnification TEM images. The black and white columns represent the numbers of living and dead *L. pneumophila* for the three TEM images, respectively. Significance was analyzed by the chi-square test. Asterisks indicates the statistical difference (*** $p < 0.001$). (**E**) Tier of *L. pneumophila* in each group.

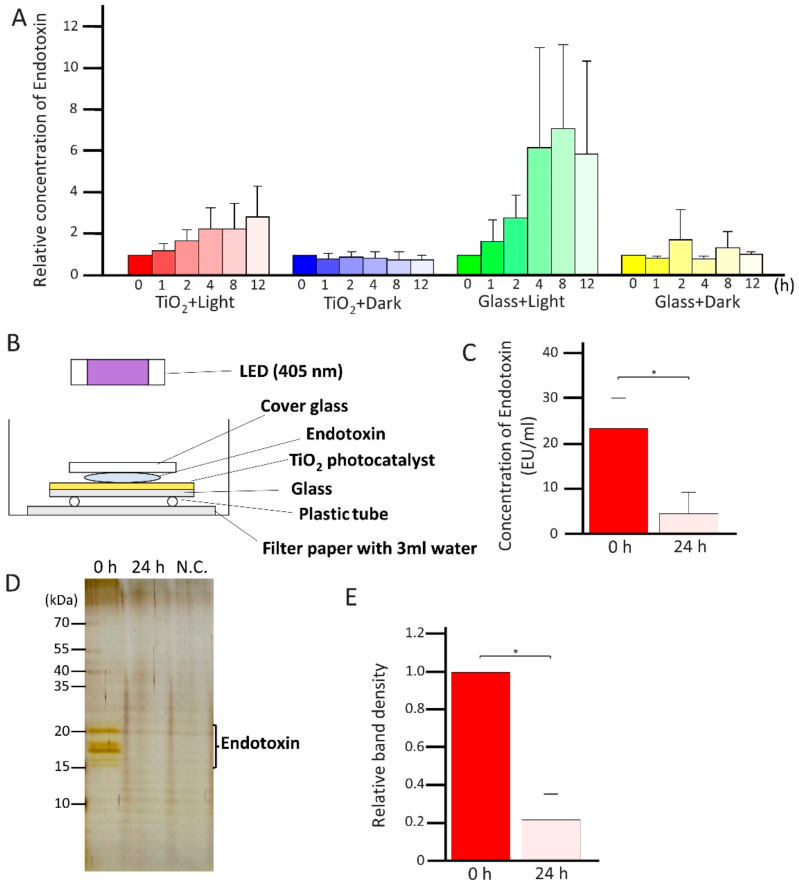

Figure 5. Release and degradation of *L. pneumophila* endotoxin by the TiO_2 photocatalyst. (**A**) In the "TiO_2 + Light" group, 30 mL of *L. pneumophila* with a titer of 1×10^7 CFU/mL was added to a 10-cm diameter dish with TiO_2-coated glass and exposed to a LED light with a wavelength of 405 nm. Samples (1 mL) were taken at the indicated intervals. During irradiation, the dish was shaken at 40 rpm. As a control, *L. pneumophila* was incubated with either TiO_2-coated glass without LED light ("TiO_2 + Dark" group), glass and LED light ("Glass + Light" group), or glass without LED light ("Glass + Dark" group). The concentration of endotoxin in each collected sample was measured using Limulus color KY. Significance between 0 min and other time points in each group was determined using two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. (**B**) Schematic diagram of degradation of endotoxin by the TiO_2 photocatalyst. (**C**) Concentration of endotoxin was measured using Limulus color KY. Significance between 0 and 24 h was determined using the Student's *t*-test. (**D**) Endotoxin was detected by silver staining. Positions of endotoxin and corresponding molecular weights are indicated. (**E**) Intensities of bands were analyzed using the ImageJ software. Significance between 0 and 24 h was determined using the Student's *t*-test. The asterisk indicates the statistical difference (* $p < 0.05$).

2.6. Durability of TiO_2 Photocatalyst

To check the integrity of the TiO_2-coated glass before and after photocatalysis, we used SEM to compare the surfaces of the glass plate without TiO_2, the fresh TiO_2-coated glass plate and the TiO_2-coated glass plate after they had been used for the photocatalytic tests (Figure 6A). The SEM image of the frost glass plate without TiO_2 exhibited a uniformly uneven surface (Figure 6A). In contrast, the SEM image of the glass plate coated with TiO_2 revealed the surface to be covered with small particles and uniform unevenness was not observed (Figure 6A). These results suggested that TiO_2 completely covers the surface of the ground glass. In addition, TiO_2 did not detach from TiO_2-coated glass plate used for photocatalytic disinfection for 24 h (Figure 6A). Moreover, to confirm the amounts of TiO_2 on the glass, elemental analysis was performed using energy dispersive X-ray analyzer

(EDS) (Figure 6B). The elemental analysis results showed that there was no significant difference between the amounts of TiO_2 (wt%) on the glass before and after use (Figure 6C). This result also strongly suggested that TiO_2 did not detach during the sterilization process. To confirm the activity of the TiO_2 photocatalyst before and after use for sterilization, methylene blue degradation assay was performed (Figure 6D). There was no difference between the rate of the TiO_2 photocatalyst-mediated degradation of methylene blue before and after use for sterilization. These results suggested that the TiO_2-coated glass in this study could be re-used and would retain activity for a long time.

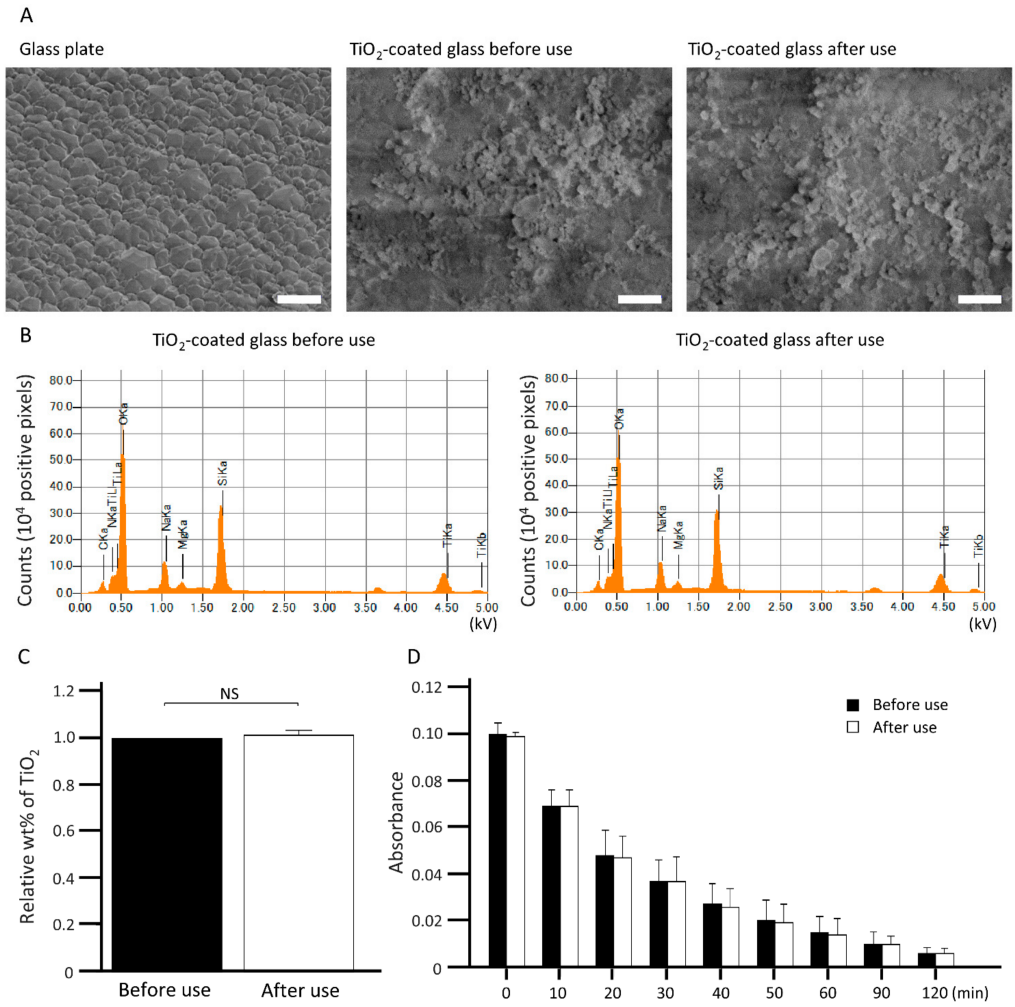

Figure 6. SEM images, elemental analysis, and methylene blue degradation of TiO_2-coated glass before and after use. (**A**) The SEM images shows surface of frosted glass plate, TiO_2-coated glass before use and after use. Bar = 500 nm. (**B**) Elemental analysis on the surface of the TiO_2-coated glass before use and after use was performed using an energy dispersive X-ray analyzer (EDS). Histogram show counts of positive pixels at each voltage. The peak of each chemical elements is indicated. (**C**) The amounts of TiO_2 on the glass (wt%) was calculated from the EDS result. Each column and error bar represent the mean ± standard deviation (SD) for two images in two independent experiments. Significance of the difference between the amounts of TiO_2 on the glass before use and after use was determined using the Student's *t*-test. NS indicates not significant. (**D**) TiO_2-coated glass before and after use in 100 mL of 12.5 nM methylene blue in water purified by ion-exchange was irradiated with a light emitting diode (LED) with a wavelength of 405 nm; absorbance was calculated at 660 nm from 0 to 120 min. Each column and error bar represent the mean ± SD for three experiments.

3. Discussion

In this study, we first confirmed the efficient disinfection of both *E. coli* and *L. pneumophila* in water by a TiO_2 photocatalyst coated on glass. Our results confirm the sterilization ability of TiO_2-coated glass against two kinds of Gram-negative bacteria, suggesting that TiO_2-coated glass might be effective for all Gram-negative bacteria in water. This supports a previous study that showed that TiO_2 photocatalysts can kill many kinds of microorganisms [4]. However, the present study is the first to confirm by TEM imaging that there was membrane damage to *L. pneumophila*, suggesting that this is the likely mechanism of disinfection during the TiO_2 photocatalytic reaction. Moreover, our results also demonstrate endotoxin release from *L. pneumophila*. Photocatalytic degradation of the endotoxin, which is a novel finding, was confirmed by LAL assay and silver stain. Degradation of the endotoxin accompanied by morphological changes to *L. pneumophila* following the TiO_2 photocatalyst reaction was confirmed by TEM imaging. The morphological changes to *L. pneumophila* included membrane separation from the cytoplasm, membrane damage (e.g., lacking the fluffy edge), and no stained cytoplasm. As a result, the density of staining of the cytoplasm was reduced. Previous reports showed similar membrane damage in *E. coli* and *Pseudomonas aeruginosa* induced by TiO_2 photocatalytic reactions [45,46]. In addition, the membrane of *E. coli* was degraded by ROS produced by the TiO_2 photocatalytic reaction, leading to the leakage of intracellular molecules, and causing cell death [44,47].

The concentration of endotoxin increased in a time-dependent manner following exposure to excitation light with TiO_2-coated glass. Endotoxin, which is a component of the outer membrane of Gram-negative bacteria, is a causative agent of sepsis. It was previously reported that antibiotics induce endotoxin release during disinfection [43]. Furthermore, endotoxin release is also caused by an increase in the permeability of the membrane [48]. Moreover, TiO_2 photocatalysts can increase the permeability of cancer cell membranes [49]. Indeed, our TEM results clearly demonstrate morphological changes of *L. pneumophila* induced by TiO_2 photocatalytic disinfection. These previous reports and our results suggest that increase in endotoxin concentration was due to endotoxin release from *L. pneumophila* via membrane damage by the TiO_2 photocatalyst in water. However, we found that the increase in endotoxin concentration by the TiO_2 photocatalyst was limited compared with that induced by disinfection by 405 nm LED without the TiO_2 photocatalyst. This suggests the possibility for TiO_2 photocatalytic degradation of endotoxin released from *L. pneumophila* in water. Until now, no report has shown the degradation of endotoxin of *L. pneumophila* by photocatalyst; however, it has been reported that endotoxin extracted from *E. coli* was degraded by a TiO_2 photocatalyst [9,10]. Here, we directly confirmed the degradation of endotoxin extracted from *L. pneumophila* by LAL assay and silver staining. Since the oxidation of high molecular weight compounds is complicated and the photocatalytic degradation order of reaction of the endotoxin has not yet been clearly revealed, it remains unclear whether *L. pneumophila* endotoxin treated with a TiO_2 photocatalyst loses virulence with degradation. Therefore, further study (for example, experimental inoculation of mice with endotoxin treated with or without the TiO_2 photocatalyst) is needed to clarify the TiO_2 photocatalytic effect on the endotoxin and loss of virulence.

The 405 nm LED without the TiO_2 photocatalyst also decreased the titer of both *E. coli* and *L. pneumophila*. This suggests that 405 nm LED also has an anti-bacterial ability. It is known that 405 nm light stimulates porphyrin molecules to produce ROS and damages cells leading to microbial death [49,50]. Likewise, it has been reported that some bacteria, such as *E. coli* and *Legionella rubrilucens*, which is closely related to *L. pneumophila*, were disinfected by 405 nm light [50]. In this study, endogenous porphyrin was excited by the 405 nm LED. This is an advantage of the TiO_2 photocatalyst, as it can disinfect microorganism by two mechanisms.

Numerous studies have considered the disinfection of *L. pneumophila*, with methods including UV, chlorination, heat, and filtration [46]. Each method has advantages and disadvantages. For example, UV is harmful to the human body. *L. pneumophila* infection

can have various sources such as contaminated showerheads [22], humidifiers [29], dental unit waterlines [30], or hot springs [31–35]; therefore, chemical compounds such as sodium hypochlorite and antibiotics are not suitable for disinfection. Physical treatment such as heat and filtration offer only short-term benefits; *L. pneumophila* propagates again after sterilization. In the case of TiO_2 photocatalyst, TiO_2 is not cytotoxic and is regarded as low risk for causing cancer [1–3]. In addition, 405 nm LED which can excite rutile-type TiO_2 photocatalyst is also harmless, unlike UVC, and can be applied even in places where sunlight does not reach. Moreover, in this study, the TiO_2 was retained on the glass plate after the photocatalytic reaction, suggesting that the TiO_2 photocatalyst can be re-used. Due to the safety and durability of the TiO_2 photocatalyst and 405 nm LED, TiO_2 photocatalyst can be applied for continuous disinfection of living and working spaces, without needing to evacuate people. It is expected that photocatalysts will be especially useful when applied to humidifiers, which are one of the main sources of *L. pneumophila*, and to hot spring pipes, in which *L. pneumophila* grows and where it is difficult for people to clean regularly. Thus, we suggest that TiO_2 photocatalysts offer great promise in effectively controlling *L. pneumophila* transmission.

4. Materials and Methods

4.1. Preparation of TiO_2-Coated Glass or Glass Fiber Sheet

TiO_2 was coated to the frosted glass plate (SAG-003, Saint-Gobain S.A., Courbevoie, France) or glass fiber sheet (XU1310010, Osaka Lighting Corp., Osaka, Japan) by the following method. Commercial powder of fine (~20 nm) rutile-type TiO_2 containing approximately 1% platinum dioxide to improve the photocatalytic reaction (MPT-623, Ishihara Sangyou Kaisha, Ltd., Osaka, Japan) [51,52] was dispersed in ion-exchanged water, and then, a frosted glass plate or a glass fiber sheet was immersed. To fuse the TiO_2 and glass, the glass plate or fiber was dried in air at room temperature before being calcined in air at 400 °C for 90 min. Typically, 26 mg TiO_2 was coated on the glass plate.

4.2. Methylene Blue Degradation

A glass plate (5 cm x 5 cm) coated with TiO_2 was put in 100 mL of 12.5 nM methylene blue solution, and an LED light (405 nm) source was placed 1.5 cm above the TiO_2-coated glass. The TiO_2 photocatalyst was excited by the 405 nm LED light, for 0, 10, 20, 30, 40, 50, 60, 90, and 120 min. To confirm the effect of TiO_2-LED on the methylene blue, methylene blue was collected at each time point, and absorbance at 660 nm was measured using an ASV11D-H spectrophotometer (AS ONE CORPORATION, Osaka, Japan). As a control, methylene blue was incubated with TiO_2-coated glass without light, with glass and LED light, and with glass without light.

4.3. Scanning Electron Microscopy of TiO_2-Coated Glass

To observe the surface of the TiO_2-coated glass plate, a scanning electron microscopy (SEM) image was obtained using a VHX-D510 electron microscope (KEYENCE CORPORATION, Osaka, Japan) at 1.2 kV.

4.4. Elemental Analysis

To confirm the amounts of the TiO_2 on the surface of glass, elemental analysis was performed using SEM with a dispersive X-ray analyzer (EDS) (JSM-IT100, JEOL Ltd., Akishima, Japan) at an accelerating voltage of 0.0 to 9.0 kV.

4.5. Microorganisms

L. pneumophila was obtained from RIKEN BRC (Tsukuba, Japan) and grown in buffered charcoal yeast extract (BCYE; 1% ACE buffer (pH = 6.9), 1% yeast extract, 0.2% charcoal, 0.1% α-ketoglutaric acid, 0.04% L-cysteine, and 0.025% ferric pyrophosphate) medium at 37 °C. As a control, *Escherichia coli* (*E. coli*) XL10 gold strain was obtained from Agilent Technologies, Inc. (Santa Clara, CA, USA) and grown in Lysogeny Broth (LB; 1% Tryptone,

1% NaCl, and 0.5% yeast extract) at 37 °C. To measure colony forming units (CFU) as a titer of *L. pneumophila* and *E. coli*, 10 to 100 μL of each suspension were transferred onto an LB agar plate or BCYE agar plate using a plate spreading technique and then incubated for 1 and 3 days at 37 °C, respectively.

4.6. Treatment of E. coli and L. pneumophila by the TiO_2 Photocatalytic Reaction

TiO_2-coated glass (5 cm × 5 cm) was placed in a dish with a diameter of 10 cm, and an LED light (405 nm) source was placed above the dish. *E. coli* in an LB medium was centrifuged at 3000 rpm for 10 min, and the pellet was resuspended in PBS. The *L. pneumophila* was centrifuged at 3000 rpm for 10 min, and the pellet was resuspended in PBS. Then, 30 mL of the suspension of either *E. coli* with 10^8 CFU/mL or *L. pneumophila* with 10^7 CFU/mL was added to a 10-cm diameter dish with TiO_2 photocatalyst coated glass and was excited by a 405 nm LED for 0, 1, 2, 4, 6, 8, 10, and 12 h. During excitation, 1 mL of the suspensions of *E. coli* and *L. pneumophila* were collected at each time point. As a control, *E. coli* and *L. pneumophila* were incubated with TiO_2-coated glass without light, with glass and LED light, and with glass without light. During irradiation, the dish was shaken at 40 rpm. A portion of the collected suspension was used to measure CFU as the titer of *E. coli* or *L. pneumophila*. The rest of the suspension of *L. pneumophila* was used to measure the concentration of endotoxin by the LAL assay.

4.7. LAL Assay

The concentration of endotoxin was measured using Limulus color KY (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) according to the manufacturer's instructions. Briefly, 50 μL of each sample was mixed with 50 μL of the Limulus Amebocyte lysate reagent in the Limulus Color KY into a 96-well plate, and absorbance was measured at 405 nm every 5 min. The levels of endotoxin were measured by comparing with a standard endotoxin solution

4.8. TEM

We placed 30 mL of *L. pneumophila* with 2.91×10^7 CFU/mL in a 10-cm diameter dish with the TiO_2-coated glass (5 cm × 5 cm). The photocatalytic reaction was activated by exposure to LED light (405 nm) for 24 h. As a control group, *L. pneumophila* was collected immediately before irradiation. After incubation, 100 μL of *L. pneumophila* was mixed with 100 μL of 2.5% glutaraldehyde for TEM negative staining. For TEM sample preparation, a droplet of *L. pneumophila* sample was loaded on a carbon-film grid and incubated for 10 s. Next, the grid was partially dried, and a droplet of 2% uranyl acetate staining solution was added, followed by incubation for 10 s. Finally, the excess liquid was removed with filter paper, and the grid was dried at room temperature before obtaining images using a HITACHI H-7600 electron microscope (Hitachi Global Life Solutions, Inc., Tokyo, Japan) at 100 kV.

4.9. Extraction of Endotoxin from L. pneumophila

Endotoxin from *L. pneumophila* with 8×10^{10} CFU was extracted by the hot phenol-water method using a lipopolysaccharide extraction kit (iNtRON Biotechnology; Seongnam, Korea) according to the manufacturer's instructions. Extracted endotoxin was redissolved in 10 mM Tris-HCl (pH = 8.0), and its concentration was measured by the LAL assay.

4.10. Inactivation of Endotoxin from L. pneumophila by the TiO_2 Photocatalytic Reaction

To confirm the degradation of extracted endotoxin from *L. pneumophila* by TiO_2 photocatalyst, extracted endotoxin was treated by a TiO_2-coated glass sheet as follows. Filter paper was placed at the bottom of the 10-cm diameter dish and moistened with 3 mL sterilized water. To avoid directly touching the filter paper, the plastic tube was placed on the filter paper, and the glass sheet coated (1 cm × 1 cm) with TiO_2 photocatalyst was put on it. Then, 100 μL of extracted endotoxin from *L. pneumophila* with a titer of 100 EU/mL

was dropped onto the glass sheet with TiO_2 photocatalyst and covered with glass. The sample then was illuminated with 405 nm LED for 24 h. After illumination, the sample was immersed and washed by 100 μL 10 mM Tris-HCl (pH = 8.0).

4.11. Silver Stain

We mixed 12 μL of endotoxin treated with the photocatalyst with 4 μL of sample buffer (0.15 M Tris-HCl, 10% sodium dodecyl sulfate [SDS], 30% glycerol, 5% beta-mercaptoethanol, and 0.5% bromophenol blue) and heated at 100 °C for 5 min. Then, 15 μL of denatured endotoxin was loaded on a 15% SDS-polyacrylamide gel and electrophoresed with a running buffer containing 0.3% Tris, 0.1% SDS, and 1.44% glycine. The endotoxin was then detected by the silver staining method using a silver stain MS kit (FUJIFILM Wako Pure Chemical Corporation) according to the manufacturer's instructions.

4.12. Statistical Analysis

Two-way ANOVA with Dunnett's test was used to compare all samples with the sample obtained at 0 min for statistical determination. To compare each group, ANOVA followed by Tukey's test was performed. p values < 0.05 were considered statistically significant. For TEM data analysis and endotoxin concentration analysis, the Student's *t*-test was used to compare the 0 and 24 h samples. In addition, Student's *t*-test was used to compare the amounts of TiO_2 on the glass before and after use in the elemental analysis. Exponential regression analysis and linear expression analysis was performed to determine the relationship between absorbance of methylene blue and irradiation time and between disinfection effect of *E. coli* and *L. pneumophila* and irradiation time. All calculations were performed using the R software (version 3.6.3, R Foundation for Statistical Computing, Vienna, Austria).

5. Conclusions

To the best of our knowledge, this is the first report to show that TiO_2 photocatalyst can degrade the membrane and endotoxin of *L. pneumophila*. TEM imaging showed TiO_2 photocatalyst caused morphological changes in *L. pneumophila,* including separation of the membrane from the cytoplasm, and reduced staining of the cytoplasm, suggesting that the primary disinfection mechanism of *L. pneumophila* is membrane degradation and leakage of cellular components. In summary, the TiO_2 photocatalyst can efficiently disinfect *L. pneumophila* (half-value period: 6.7 min), without harm to people. In addition, the TiO_2 photocatalyst degrades the endotoxin of *L. pneumophila* in water. In conclusion, the TiO_2 photocatalyst could be an effective tool for controlling the spread of *L. pneumophila*.

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