Studies on photokilling of bacteria on TiO\textsubscript{2} thin film

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Abstract

In order to elucidate the mechanism for photokilling of \textit{Escherichia coli} (\textit{E. coli}) cells on titanium dioxide (TiO\textsubscript{2}) thin film, the survival of intact cells and the spheroplasts was investigated as a function of photo-illumination time. The photokilling reaction for intact cells was observed to involve two steps, an initial lower rate photokilling step followed by a higher rate one. In contrast, the reaction for spheroplasts, which do not have cell wall, exhibited only a single step kinetics with a higher rate, suggesting that the cell wall of \textit{E. coli} cell acts as a barrier to the photokilling process. Changes in concentration of the cell wall components during illumination further showed that the outer membrane serves as a barrier, while the peptidoglycan layer does not have a barrier function. Moreover, atomic force microscopy measurements of cells on illuminated TiO\textsubscript{2} film showed that the outer membrane decomposed first, and with further illumination, the cells completely decomposed. These results suggest that the photokilling reaction is initiated by a partial decomposition of the outer membrane, followed by disordering of the cytoplasmic membrane, resulting in cell death.

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1. Introduction

Purification of environmental toxic substances in water and air using TiO\textsubscript{2} photocatalysts has been studied extensively [1–8]. In many studies on water treatment, for example, a fine TiO\textsubscript{2} powder was suspended in treatment water and illuminated with a strong light, such as a mercury lamp. This system, however, had two problems, i.e. the recovery of TiO\textsubscript{2} powder and the high cost of light. We have focused our attention on the photocatalytic reactions of TiO\textsubscript{2} thin films coated on various substrates under rather weak ultraviolet (UV) light intensity. These films showed deodorizing, bactericidal, and self-cleaning functions by absorbing UV light existing in the ordinary living environment [7–21]. In fact, TiO\textsubscript{2} coated tiles with self-cleaning and bactericidal function have already been commercialized [9,10]. The self-cleaning function of TiO\textsubscript{2} coated substrates is explained simply by the photo-induced oxidative power of TiO\textsubscript{2} photocatalysts. The bactericidal function, however, is not well understood, even though numerous reports have described photokilling of bacteria [20–32], viruses [33–35] and tumor cells [11,36–39]. Because these photokilling reactions were carried out using TiO\textsubscript{2} powder, the possibility of cell deactivation by the co-aggregation of cells and TiO\textsubscript{2} particles cannot be excluded. In addition, the TiO\textsubscript{2} particles phagocytosed by the cells may cause cellular injury [36]. In fact, Jacob and coworkers reported that TiO\textsubscript{2} particles ingested by phagocytosis cause rapid intracellular damage [32].

We reported the photokilling activity of \textit{E. coli} on a TiO\textsubscript{2} thin film [20]. In this case, the cell survival was only affected by damage from the outside of the cell, by a photocatalytic reaction. This allowed for evaluation of survival based solely on photocatalysis. We also showed that the TiO\textsubscript{2} film photocatalyst decomposes lipopolysaccharide (LPS), which is a pyrogenic constituent of the \textit{E. coli} cell wall [21]. These results showed that the antibacterial effect of TiO\textsubscript{2} coated materials is not a simple bacteriostatic action, but instead a bactericidal action, which involves decomposition of the cell wall. In the present report, we investigate the survival of \textit{E. coli}\textsubscript{t} on TiO\textsubscript{2} film as a function of UV illumination time, and describe observations from atomic force microscopy (AFM).

2. Experimental

TiO\textsubscript{2} thin films were prepared by a conventional dip-coating technique. Silica-coated soda-lime glass plates were dipped in a commercially available titanium isoproxide solution (NDH-510C, Nippon Soda), and subsequently
withdrawn from the solution at a fixed rate of 20 cm/min. The plates were then placed in a furnace and calcined at 500°C for 1 h. Four such coating steps produced a TiO₂ thin film with a thickness of ∼0.4 μm on both sides of the glass plate.

Photocidal activity of the illuminated TiO₂ film was evaluated as follows. E. coli cells (IFO 3301 strain) were precultured in a 2.5 ml nutrient broth (“Daigo”, Nippon Seiyaku) at 30°C for 18 h, and then washed by centrifuging at 4000 rpm. The treated cells were then re-suspended and diluted to ∼2 × 10⁵ colony forming units (CFU)/ml with sterilized water. The diluted cell suspension was pipetted onto a TiO₂-coated glass plate, and placed in an air-tight illumination chamber to prevent drying (Fig. 1). The chamber was illuminated with a 15-W black-light bulb (Type FL15 BL-B, National Panasonic) at a light intensity of 1.0 mW/cm². The light intensity was measured using a UV radiometer (UVR-36, Topcon). After being illuminated for a certain period of time, the cell suspension, which retained water droplets due to the air-tight chamber, was collected into a 0.15 M saline solution. An appropriate dilution of the collected cells was incubated at 36°C for 24 h on a nutrient agar medium (Standard Method Agar “Nissui”, Nissui Seiyaku) to determine the number of viable cells in terms of CFU. The same evaluations were also performed using higher initial cell concentrations (2 × 10⁶ CFU/ml, 2 × 10⁷ CFU/ml, 2 × 10⁸ CFU/ml).

Spheroplasts (10⁸ CFU/ml level) were prepared by treating the intact E. coli cell suspension with 0.03 M Tris–HCl buffer (pH 8.1) containing 20% sucrose with lysozyme (1 mg/ml) and EDTA (ethylenediamine tetraacetate, 0.1 M pH 7.0) at 30°C for 60 min [40]. The treated cells were separated by centrifugation, and re-suspended in an 0.2 M phosphate buffer (pH 6.6) containing 0.2% MgCl₂. The spheroplast suspension was illuminated under the same conditions as the intact cells. The survival of the spheroplasts and the corresponding intact cells was determined using Live/Dead BacLight Bacterial Viability Kits (Molecular Probes Inc.), which can detect bacterial survival by a two-color fluorescence assay.

To determine the change in LPS concentration, the E. coli cell suspension on the TiO₂-coated glass was diluted with pyrogen-free water after illumination. The Limulus test, which is a colorimetric method (Toxicolor Test, Seikagaku Kogyo), was performed for the appropriate dilutions. A standard endotoxin solution (E. coli O111: B4, Seikagaku Kogyo) was used as the test. The assay of peptidoglycan concentration was performed with the SLP (Silkworm Larvae Plasma) reagent set (Wako Pure Chemicals) with Toxinometer ET-301 (Wako Pure Chemicals) [41,42]. The PG solution refined from Micrococcus luteus (Wako Pure Chemicals) was used as a standard in this assay.

E. coli cells on TiO₂ film before and after UV light illumination were observed directly under ambient conditions by a commercial AFM system (SPA300, Seiko Instruments) using triangular Si₃N₄ sharpened cantilevers with a force between the tip and the sample of 1 nN. The cell suspension dropped on the TiO₂ film was at a concentration of 2 × 10⁸ CFU/ml.

3. Results and discussion

Fig. 2 shows the survival of intact E. coli cells dropped on various substrates as a function of time. No obvious changes in survival were observed when the TiO₂ coated substrate was stored in the dark or when a conventional soda-lime glass plate was used. The cell suspension dropped on the TiO₂ film was at a concentration of 2 × 10⁸ CFU/ml.

Fig. 2. The log plot of the survival of E. coli cells vs. illumination time. The cell suspension was incubated on TiO₂ film under UV illumination (1.0 mW/cm²). The initial cell concentrations were 2 × 10⁵ CFU/ml ( ), 2 × 10⁶ CFU/ml ( ), 2 × 10⁷ CFU/ml ( ), 2 × 10⁸ CFU/ml ( ), 2 × 10⁹ CFU/ml ( ), respectively. Survival was also determined for cells (2 × 10⁵ CFU/ml) on the TiO₂ film in the dark ( ); and on normal glass (soda-lime glass, SLG) without TiO₂ film under UV illumination ( ) (1.0 mW/cm²). The data shown in the figure are the average values of three experiments.
glass (SLG) was used as the substrate under UV illumination. In contrast, a great decrease in the number of viable cells was observed on the illuminated TiO$_2$ film, demonstrating its photokilling activity. When the initial cell concentration was $2 \times 10^5$ CFU/ml, cell killing was completed within only $\sim 90$ min under the present experimental conditions.

It should be emphasized that the survival curve does not follow a simple single exponential decay process as a function of illumination time, but seems to consist of two steps, a very low rate photokilling step, followed by a higher one. Such two-step decay dynamics was observed regardless of the initial cell concentration in the range of $2 \times 10^5$–$2 \times 10^8$ CFU/ml. In the case of an initial cell concentration of $2 \times 10^7$ CFU/ml, the rate constants of the first and second steps were 0.015 and 0.085 min$^{-1}$, respectively, which are close to those obtained in the powder systems [27,33]. Several contradictory reports have been published on the decay dynamics of the photokilling process of microbes. One reported that decay follows a pseudo first-order kinetics [27,33], while another report showed that it follows a model profile based on a series-event model or a single-hit multi-target model [26,29]. The profile of the latter decay curve is similar to that of our two-step decay one, suggesting that our decay curve could be also fitted to their models. However, because we would like to emphasize the existence of the slow decay step, we used the two-step decay model. Furthermore, we used TiO$_2$ film as a photocatalyst, not TiO$_2$ powder. In the case of TiO$_2$ powder, as was described previously, simple cell co-aggregation with TiO$_2$ particles could decrease cell survival, even in the dark [24]. In contrast, the survival on the film photocatalyst under dark conditions was almost 100% during the experiments. Therefore, we believe that the present two-step dynamics reflects the actual photokilling process.

Next, we studied the survival change of E. coli spheroplasts. The cell envelope of E. coli consists of the following three layers (starting from the inside and moving outward): a cytoplasmic membrane, a monolayer of peptidoglycan, and an outer membrane. Spheroplasts lack the peptidoglycan layer and part of the outer membrane of the cell envelope. Fig. 3 shows the results on spheroplasts as well as on intact cells under the same conditions. Unlike the survival of intact cells, spheroplasts showed a simple single exponential decay dynamics, with a higher rate constant than that for the intact cells. The cell wall (the outer membrane and the peptidoglycan layer) may block the reactive species (e.g. $h^+$ (hole), $\bullet OH$, $O_2^{-}$, $H_2O_2$) produced by TiO$_2$ photocatalysis. Therefore, these data suggest that the cell wall of the intact cell is damaged during the initial step with the lower photokilling rate.

The outer membrane, the outermost layer of the cell wall, contains phospholipid, protein, and LPS as major constituents [43,44]. To show direct damage of the cell wall, we measured the concentration of LPS in the outer membrane and peptidoglycan in the peptidoglycan layer. Fig. 4 shows the concentrations of LPS and peptidoglycan as well as the survival of E. coli in the intact cell suspension. Interestingly, the concentration of the LPS increased slightly during the initial slow step of intact cell killing. A similar increase was reported when bacteria were killed using antibiotics [45,46]. Such an increase suggests that the outer membrane is decomposed by photocatalysis and that LPS is liberated from the outer membrane of the cells, because the reactivity of liberated LPS with the Limulus reagent is higher than that of the fixed LPS [47]. By the subsequent illumination, the LPS concentration decreased, indicating that the free LPS and probably the fixed LPS as well are decomposed by the photocatalytic reaction [21].
Fig. 5. AFM images of \textit{E. coli} cells on the TiO\textsubscript{2} film: (a) no illumination, (b) illumination for 1 day, (c) illumination for 6 days. Light intensity was 1.0 mW/cm\textsuperscript{2}.
Fig. 6. (a–c) Schematic illustration of the process of E. coli photokilling on TiO$_2$ film. In lower row, the part of cell envelope is magnified.
In contrast, the concentration of peptidoglycan in the peptidoglycan layer located inside the outer membrane, did not change during this time frame, under the same illumination conditions. It started decreasing after 6h of illumination (data not shown). Since the peptidoglycan concentration was determined using the SLP reagent set, the decrease in concentration indicates that the peptidoglycan decomposes to the approximate molecular size of MDP (muramyl dipeptide) [41,42]. Therefore, the absence of any change in peptidoglycan concentration suggests that photokilling proceeds even without peptidoglycan decomposition to a size as small as MDP. These data indicate that the outer membrane acts as a barrier to the reactive species produced by photocatalysis, and that the cell is not easily deactivated during the initial illumination period. However, when the barrier decomposes, cell viability is easily lost.

Fig. 5 shows AFM photographs of E. coli cells on TiO₂ film after different UV illumination times. Before illumination, the cells have a cylindrical shape with sizes of ~1µm × ∼1.5–2.5µm (Fig. 5a). Even after illumination for 24h, no obvious morphological changes were recognized, even though the cells had already lost their viability by that time (Fig. 5b). The outermost layer (labeled in the figure), however, clearly observed in Fig. 5a disappeared after 24h of illumination (Fig. 5b). In contrast, the outermost layer of the cells on TiO₂ film in the dark for 24h remained intact, which was expected from that no change in the concentrations of LPS was observed (data not shown). Therefore, the disappearance of the outermost layer results from the film photocatalysis. Furthermore, the observation reflects the change in concentration of the cell wall components, and also demonstrates that cells on illuminated TiO₂ film decompose from the outside of the cell. Fig. 5c is the photograph of the TiO₂ film after 6 days of illumination. The cylindrical shape of the cells disappeared completely, suggesting the complete decomposition of dead cells.

The process of E. coli photokilling on TiO₂ film can be schematically illustrated as in Fig. 6. The initial reaction is a partial decomposition of the outer membrane by the reactive species produced by TiO₂ photocatalysis (Fig. 6b). During this process, cell viability is not lost very efficiently. The partial decomposition of the membrane, however, changes the permeability to reactive species. For example, human erythrocyte membranes treated with active oxygen species have pin holes, through which Na⁺ and glucose-6-phosphate can penetrate [48]. Correspondingly, the permeability change of the outer membrane enables reactive species to easily reach the cytoplasmic membrane. Thus, the cytoplasmic membrane is attacked by reactive species, leading to the peroxidation of membrane lipid (Fig. 6c).


diacatalyst, and suggests that a combination of TiO₂ photocatalyst with some antibacterial reagents that can penetrate the outer membrane could show a far superior photokilling activity. The use of such a combination (Cu/TiO₂) is currently under investigation.

4. Conclusion

The change in survival of intact E. coli cells on illuminated TiO₂ film demonstrates a two-step decay dynamics, the first with a lower rate constant and the second with a higher rate constant. On the other hand, decay for spheroplasts follows a single exponential decay process. The change in concentration of the cell envelope constituents (LPS and peptidoglycan) supports a two-step reaction mechanism for photokilling of intact cells. Based on these results, we propose the mechanism that the first step of the photokilling process is the disordering of the outer membrane of E. coli cells on illuminated TiO₂ film. This process is necessary for the inner membrane penetration of reactive species produced by photocatalysis. The second step of the process is the disordering of the inner membrane (the cytoplasmic membrane).

The conclusion could be obtained by using TiO₂ film photocatalyst, and suggests that a combination of TiO₂ photocatalyst with some antibacterial reagents that can permeate the outer membrane could show a far superior photokilling activity. The use of such a combination (Cu/TiO₂) is currently under investigation.

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