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Studies on photokilling of bacteria on TiO_2 thin film

Kayano Sunada [a,b], Toshiya Watanabe [a,b], Kazuhito Hashimoto [a,b,*]

[a] *Research Center for Advanced Science and Technology, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8904, Japan*
[b] *Kanagawa Academy of Science and Technology, KSP Building, 3-2-1 Sakado, Takatsu-ku, Kawasaki, Kanagawa 213-0012, Japan*

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Abstract

In order to elucidate the mechanism for photokilling of *Escherichia coli* (*E. coli*) cells on titanium dioxide (TiO_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 *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_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.

Keywords: TiO_2 film; Photocatalysis; Photokilling process; Outer membrane

1. Introduction

Purification of environmental toxic substances in water and air using TiO_2 photocatalysts has been studied extensively [1–8]. In many studies on water treatment, for example, a fine TiO_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_2 powder and the high cost of light. We have focused our attention on the photocatalytic reactions of TiO_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_2 coated tiles with self-cleaning and bactericidal function have already been commercialized [9,10]. The self-cleaning function of TiO_2 coated substrates is explained simply by the photo-induced oxidative power of TiO_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_2 powder, the possibility of cell deactivation by the co-aggregation of cells and TiO_2 particles cannot be excluded. In addition, the TiO_2 particles phagocytosed by the cells may cause cellular injury [36]. In fact, Jacob and coworkers reported that TiO_2 particles ingested by phagocytosis cause rapid intracellular damage [32].

We reported the photokilling activity of *E. coli* on a TiO_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_2 film photocatalyst decomposes lipopolysaccharide (LPS), which is a pyrogenic constituent of the *E. coli* cell wall [21]. These results showed that the antibacterial effect of TiO_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 *E. coli* on TiO_2 film as a function of UV illumination time, and describe observations from atomic force microscopy (AFM).

2. Experimental

TiO_2 thin films were prepared by a conventional dip-coating technique. Silica-coated soda-lime glass plates were dipped in a commercially available titanium isopropoxide solution (NDH-510C, Nippon Soda), and subsequently

* Corresponding author. Tel.: +81-3-5452-5080; fax: +81-3-5452-5083.
E-mail address: kazuhito@fchem.chem.t.u-tokyo.ac.jp (K. Hashimoto).

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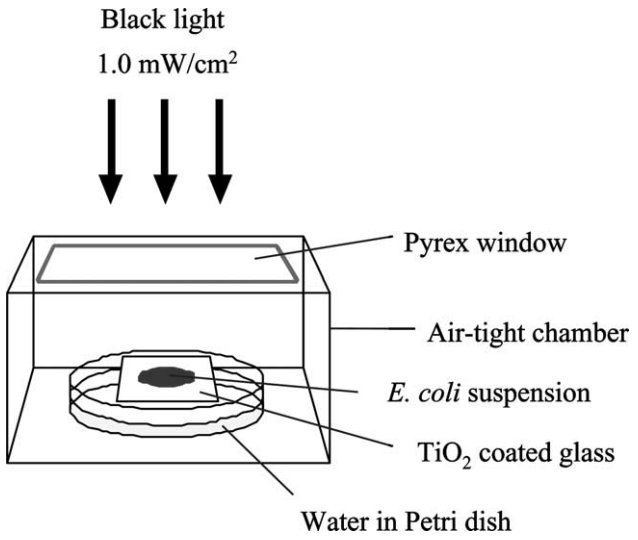

Fig. 1. Schematic illustration of the illumination system.

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 TiO_2 thin film with a thickness of ~0.4 μm on both sides of the glass plate.

Photokilling activity of the illuminated TiO_2 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 ~2×10^5 colony forming units (CFU)/ml with sterilized water. The diluted cell suspension was pipetted onto a TiO_2-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/cm^2. 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 (2×10^6 CFU/ml, 2×10^7 CFU/ml, 2×10^8 CFU/ml).

Spheroplasts (10^8 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% $MgCl_2$. 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 TiO_2-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 TiO_2 film before and after UV light illumination were observed directly under ambient conditions by a commercial AFM system (SPA300, Seiko Instruments) using triangular Si_3N_4 sharpened cantilevers with a force between the tip and the sample of 1 nN. The cell suspension dropped on the TiO_2 film was at a concentration of 2×10^7 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 TiO_2 coated substrate was stored in the dark or when a conventional soda-lime

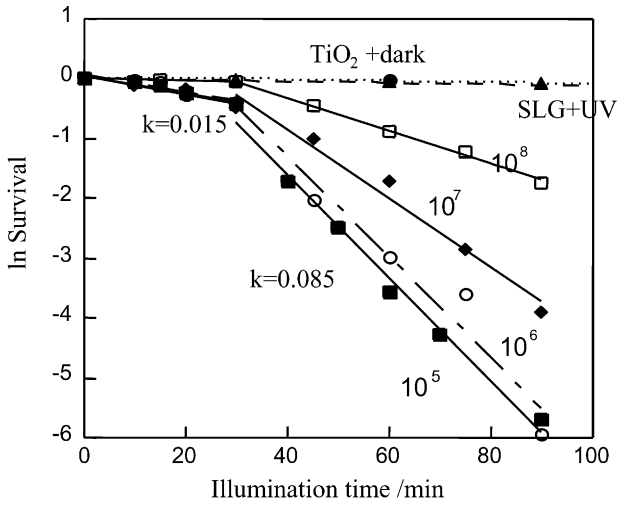

Fig. 2. The log plot of the survival of *E. coli* cells vs. illumination time. The cell suspension was incubated on TiO_2 film under UV illumination (1.0 mW/cm^2). The initial cell concentrations were 2×10^5 CFU/ml (■), 2×10^6 CFU/ml (○), 2×10^7 CFU/ml (◆), 2×10^8 CFU/ml (□), respectively. Survival was also determined for cells (2×10^5 CFU/ml) on the TiO_2 film in the dark (●), and on normal glass (soda-lime glass, SLG) without TiO_2 film under UV illumination (▲) (1.0 mW/cm^2). 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 2×10^5 CFU/ml, cell killing was completed within only ~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 2×10^5–2×10^8 CFU/ml. In the case of an initial cell concentration of 2×10^5 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 multitarget 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

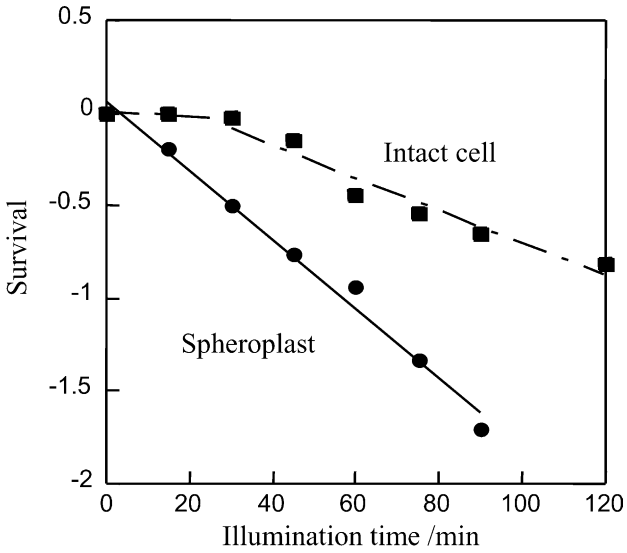

Fig. 3. Survival of the intact cells (■) and the spheroplasts (●) of *E. coli* (~10^8 CFU/ml) on TiO_2 film under UV illumination (1.0 mW/cm^2).

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].

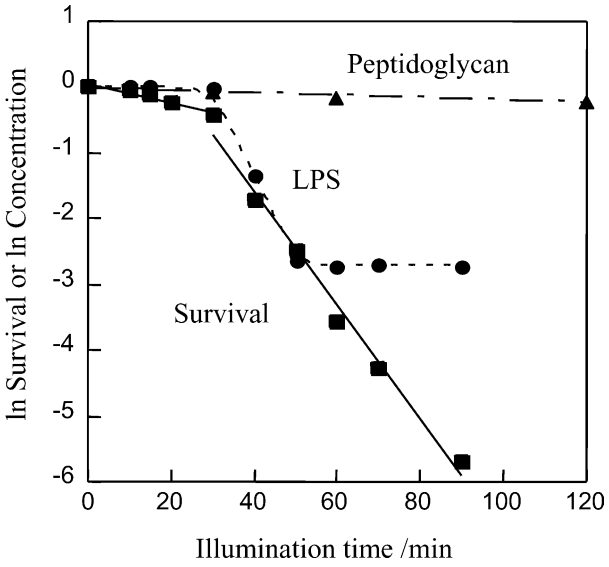

Fig. 4. Survival of *E. coli* (2×10^5 CFU/ml) (■), concentration of the LPS (●) and peptidoglycan (▲) from the cell on TiO_2 film with illumination time. Light intensity was 1.0 mW/cm^2. Initial LPS and peptidoglycan concentrations were approximately 8 EU/ml and 0.063 ng/ml, respectively.

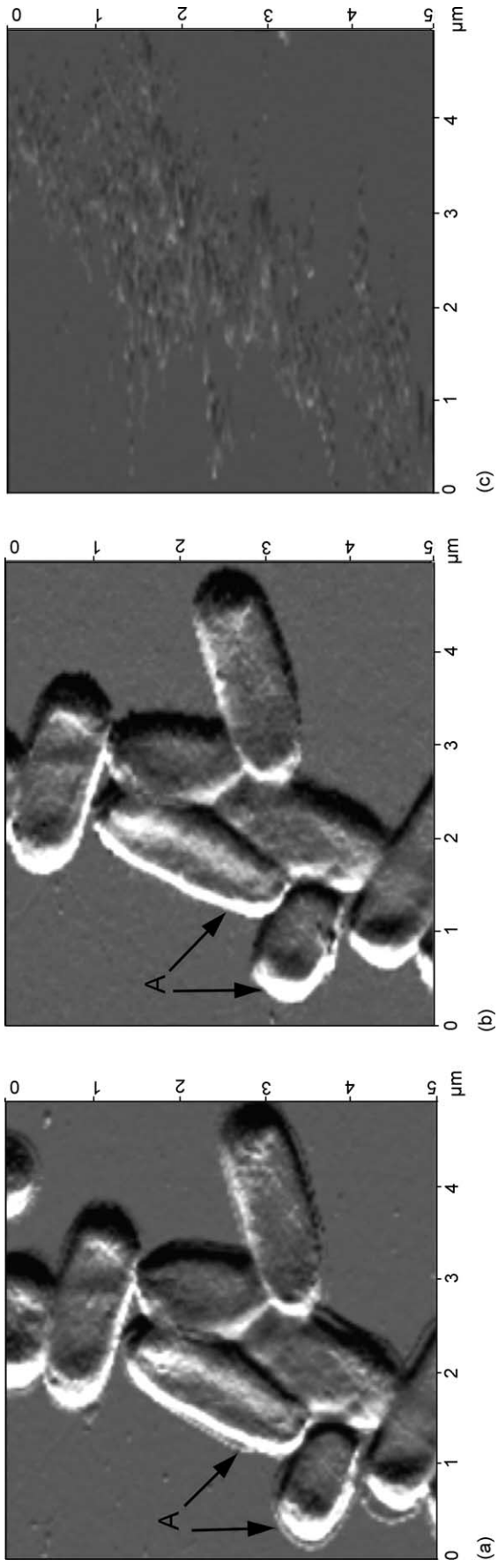

Fig. 5. AFM images of *E. coli* cells on the TiO_2 film: (a) no illumination, (b) illumination for 1 day, (c) illumination for 6 days. Light intensity was 1.0 mW/cm^2.

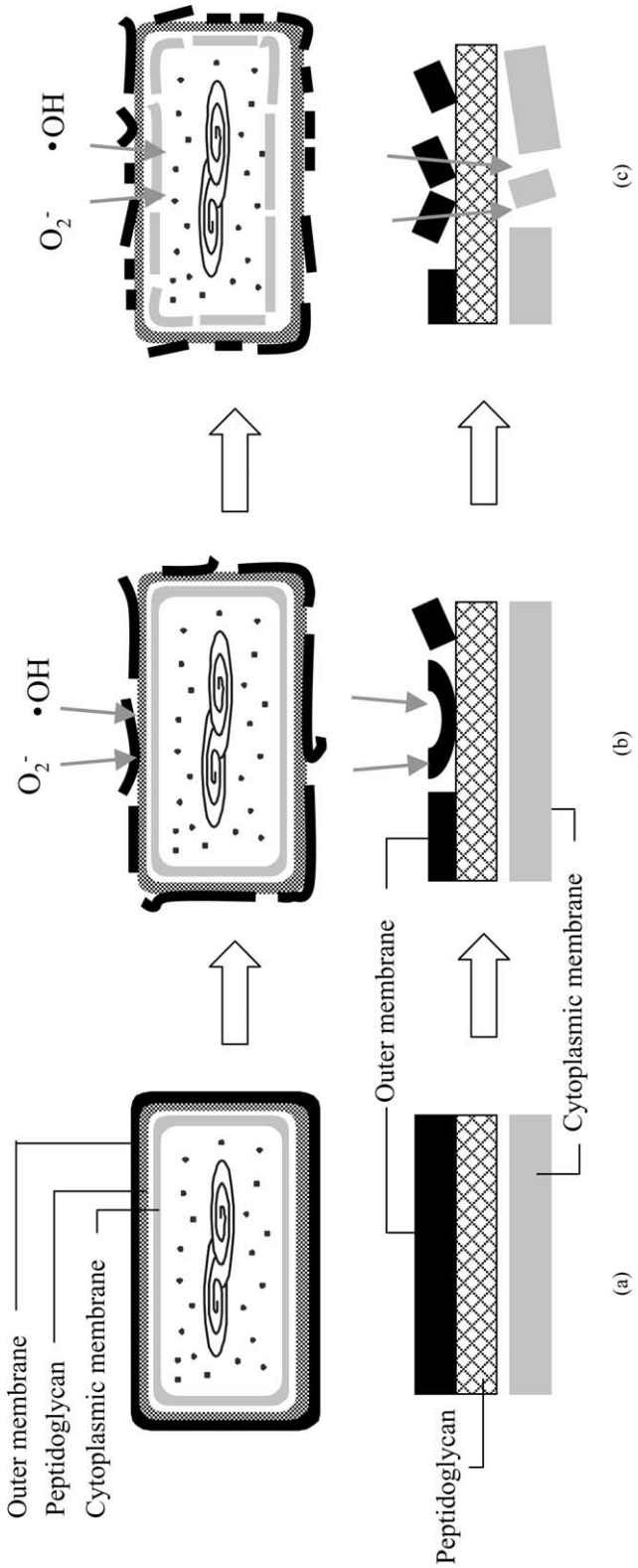

Fig. 6. (a–c) Schematic illustration of the process of *E. coli* photokilling on TiO_2 film. In lower row, the part of cell envelope is magnified.

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 6 h 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 validity is easily lost.

Fig. 5 shows AFM photographs of *E. coli* cells on TiO_2 film after different UV illumination times. Before illumination, the cells have a cylindrical shape with sizes of ∼1 μm × ∼1.5–2.5 μm (Fig. 5a). Even after illumination for 24 h, 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 24 h of illumination (Fig. 5b). In contrast, the outermost layer of the cells on TiO_2 film in the dark for 24 h 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 TiO_2 film decompose from the outside of the cell. Fig. 5c is the photograph of the TiO_2 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 TiO_2 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 TiO_2 photocatalysis (Fig. 6b). During this process, cell validity 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 penetate [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). Jacoby et al. have observed MDA (malondialdehyde) production indicating lipid peroxidation of the membrane, during treatment of *E. coli* cells with TiO_2 and UV light. The structural and functional disorders of the cytoplasmic membrane [48–50] due to lipid peroxidation lead to the loss of cell viability and cell death. Several reports support that a disorder of the cytoplasmic membrane is the root of the killing effect by TiO_2 photocatalysis [24,31,32,39]. Thus, the partial decomposition of the outer membrane in *E. coli* cells by the initial TiO_2 photocatalytic reaction, ultimately leads to cell death, showing the photokilling activity of TiO_2 film.

4. Conclusion

The change in survival of intact *E. coli* cells on illuminated TiO_2 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 TiO_2 film. This process is necessary for the inner membrane penetration of reactive species produced by photocalysis. The second step of the process is the disordering of the inner membrane (the cytoplasmic membrane).

The conclusion could be obtained by using TiO_2 film photocatalyst, and suggests that a combination of TiO_2 photocatalysts with some antibacterial reagents that can permeate the outer membrane could show a far superior photokilling activity. The use of such a combination (Cu/TiO_2) is currently under investigation.

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