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Bactericidal and Detoxification Effects of TiO_2 Thin Film Photocatalysts

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To examine the special features of the antibacterial effect for a thin transparent titanium dioxide (TiO_2) film, the photocatalytic degradation of endotoxin, which is a pyrogenic constituent of *Escherichia coli (E. coli)*, as well as its bactericidal activity, was investigated. The TiO_2 films were prepared from titanium isopropoxide solution, annealing at 500 °C. The bactericidal activity for *E. coli* cells was estimated by survival ratio calculated from the number of viable cells which form colonies on the nutrient agar plates. The endotoxin concentration was determined by the *Limulus* tests. When *E. coli* cells were killed by the TiO_2 photocatalyst under UV irradiation, the endotoxin from the cells was also degraded efficiently. This result shows that the TiO_2 photocatalyst has both bactericidal activity and decomposing activity for the endotoxin (i.e., detoxifying activity). The bactericidal effect of the TiO_2 thin film results from both inactivating the viability of the bacteria and the destruction of the *E. coli* cells. This feature renders TiO_2 photocatalysts to be applicable to environmental protections, especially in medical facilities where the endotoxin is needed to control.

Introduction

After the discovery of photoinduced water cleavage on TiO_2 electrodes (*1*), much research has been done on TiO_2 photocatalysis with the aim of developing methods to purify water and air (*2*−*5*). These reactions are based on the strong oxidizing power of TiO_2 photocatalysts under UV irradiation. We showed that TiO_2-coated materials possess deodorizing, antibacterial, and self-cleaning functions under weak, ultraviolet light in living enviroments (*6*−*9*). For the bactericidal activity, several results have been reported using TiO_2 powder (*10*−*14*), on the other hand, we applied TiO_2-coated materials for this purpose (*9*).

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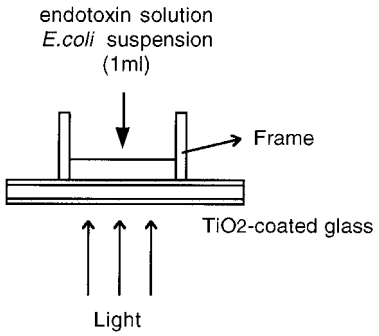

FIGURE 1. Schematic diagram of the illumination system.

In general, antibacterial reagents inactivate cell viability, but pyrogenic and toxic ingredients such as endotoxins remain even after the bacteria have been killed. Endotoxin is the lipopolysaccharide (LPS) cell wall constituent of Gram-negative bacteria and consists of a sugar chain expressed O-antigen and a complex lipid called lipid A. Lipid A plays a major role in the various types of bioactivity of endotoxins, which include not only the endotoxic activities such as pyrogenicity, lethal toxicity, and Schwartzman reactivity, but also beneficial activities such as immunoadjuvanticity and antitumor activity (*15*−*17*). However, endotoxic activities are elicited even at very low concentrations, i.e., a few nanograms per milliliter in blood, and therefore, the endotoxin causes critical problems in medical facilities and in factories manufacturing pharmaceuticals and medical devices. Because UV-irradiated TiO_2 can degrade most organic compounds by its strong oxidizing power, we here examine both the inactivation of the endotoxin and the bactericidal effect using TiO_2 film photocatalysts.

Experimental Section

TiO_2 film photocatalysts were prepared as follows. Soda-lime glass plates, previously coated with silica thin film (100 nm), were dipped in titanium isopropoxide solution (Type NTi-500, Nippon Soda) and were slowly pulled from the solution at a fixed rate of 20 cm/min in dry air. The plates were quickly placed in a furnace and calcined at 500 °C for 1 h. Four such coating steps produced a TiO_2 thin film of approximately 0.4 μm on both sides of the glass plate. The thickness was estimated from the interference oscillations in the UV−visible spectra. The TiO_2-coated glass plates were sterilized in an oven at 250 °C for 2 h to prevent endotoxin contamination.

Reagent endotoxin (*Escherichia coli* O55: B5) was used as received (Seikagaku Kogyo) and was dissolved in endotoxin-free (pyrogen-free) water.

E. coli cells (IFO 3301 strain), precultured in 2.5 mL of nutrient broth ("Daigo", Nippon Seiyaku) at 30 °C for 18 h, were washed by centrifuging at 4000 rpm and were resuspended and diluted to approximately 2×10^5 colony forming units (CFU)/mL with sterilized water.

A cylindrical frame was placed on the TiO_2-coated glass plate, and then 1 mL of the reagent endotoxin solution or *E. coli* suspension was pipetted into it. This system (Figure 1) was illuminated with a 15-W black-light bulb (Type FL15 BL-B, National Panasonic) from below. The light intensity at the working TiO_2 surface was estimated to be approximately 0.4 mW/cm^2 at the sample position, because, as it is illuminated from below, the underside of the TiO_2 layer acts as a UV-attenuating filter.

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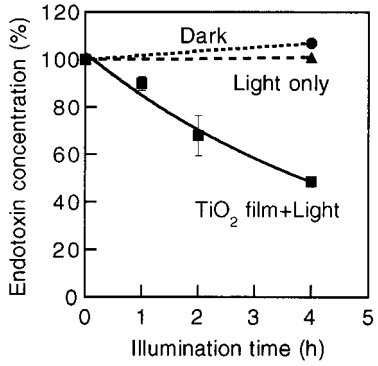

FIGURE 2. Concentration of the reagent endotoxin with (TiO_2 film + light) and without (light only) a TiO_2 thin film under black-light illumination (0.4 mW/cm^2) and with a TiO_2 thin film in the dark (dark). Initial endotoxin concentration: 1.23 EU(endotoxin unit)/mL. Error bars: standard deviations based upon three replicate experiments.

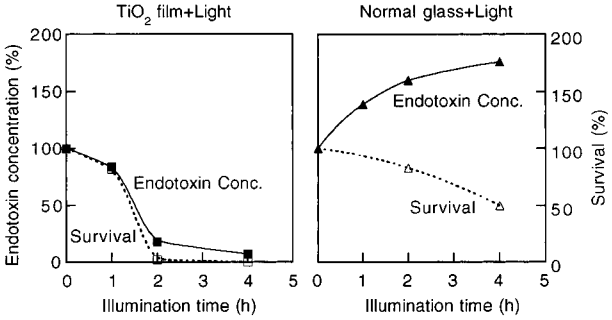

FIGURE 3. Concentration of the endotoxin exhausted from *E. coli* and survival ratio of *E. coli* under black light illumination (0.4 mW/cm^2) for TiO_2 thin film and for a normal glass (soda-lime glass) without TiO_2 film. The solid lines and dotted lines indicate the concentration of the endotoxin and the survival ratio of *E. coli*, respectively. Initial endotoxin concentration: approximately 7.4 EU/mL.

The endotoxin concentration was determined as follows. A total of 10 μL of the reagent endotoxin solution or *E. coli* suspension was sampled at timed intervals during illumination, and the *Limulus* test was performed by a colorimetric method (Toxicolor Test; Seikagaku Kogyo) to determine the endotoxin concentration by comparing a standard endotoxin solution (*E. coli* O111: B4).

The survival ratio of *E. coli* was determined by counting the number of viable cells in terms of CFU. A total of 50 μL of *E. coli* suspension was sampled, and the appropriate dilutions were plated on nutrient agar medium (Standard Method Agar "Nissui", Nissui Seiyaku) and incubated for 24 h. The details of the procedure have been described elsewhere (*9*).

Results and Discussion

First, the photocatalytic degradation of the reagent endotoxin was examined. The concentration of endotoxin on the TiO_2 thin film decreased under black light illumination. On the other hand, the concentration of endotoxin was affected neither on the TiO_2 film in the dark nor on a normal glass plate (soda-lime glass) under black-light illumination (Figure 2).

Since lipid A of the endotoxin activates a limulus coagulation factor C (*15*−*17*), the first protease in an alternative coagulation cascade of the horseshoe crab amebosites, the result obtained by the *Limulus* test indicates that lipid A is decomposed by the TiO_2 photocatalyst. For the pyrogenicity and lethal toxicity of the endotoxin, the whole structure of lipid A is necessary (*15*−*17*). Therefore, the degradation of lipid A shows that the endotoxin is inactivated by TiO_2 photocatalyst.

TiO_2 photocatalysis generates various active oxygen species as follows (*18*, *19*).
Reductive reactions:

$$O_2 + e^- \rightarrow O_2^-$$

$$O_2^- + H^+ \rightarrow \bullet HO_2$$

$$\bullet HO_2 + e^- + H^+ \rightarrow H_2O_2$$

Oxidative reactions:

$$OH^- + h^+ \rightarrow \bullet OH$$

$$\bullet OH + \bullet OH \rightarrow H_2O_2$$

In the degradation of organic compounds, the main reaction is usually the oxidation by hydroxyl radicals (•OH) (*2*). Because the lifetime of •OH is very short (*9*), main detoxification of endotoxin proceeds on the surface of TiO_2 thin film. However, it is known that lipid A of the endotoxin is also oxidized by hydrogen peroxide (H_2O_2) (*20*). Therefore, the possibility that some inactive rections proceed in the solution phase cannot be excluded in the present conditions. To clarify the mechanism of the reaction on TiO_2 surface, effects at the solid−air interface are under investigation.

Next, the concentration of endotoxin exhausted from the *E. coli* cells was examined. The survival ratio of the *E. coli* was also measured at the same time. Under the black-light illumination without TiO_2 film, the survival ratio of *E. coli* gradually decreased by photodynamic action, and simultaneously, the concentration of endotoxin increased (Figure 3). This is because the endotoxin is exhausted from the cells when they are killed (*21*, *22*). A similar phenomenon was observed when *E. coli* viability was inactivated using an antibacterial reagent such as Ag^+. Conversely, on the illuminated TiO_2 film, the concentration of endotoxin in *E. coli* suspension decreased even though the survival ratio of *E. coli* decreased. This result clearly shows that TiO_2-coated substrates can kill bacteria and also similtaneously degrade the toxic compounds exhausted from the bacteria. The decomposition of the endotoxin from the *E. coli* cells means that the TiO_2 photocatalyst destroys the outer membrane of the *E. coli* cell. All of these data show that the antibacterial effects of TiO_2-coated materials involve not only the nullification of the viability of the bacteria, but also the destruction of the bacterial cell.

Let us consider the efficiency of the degradation of the endotoxin. The reagent endotoxin decreased from 1.2 EU-(endotoxin unit)/mL to 0.84 EU/mL over 2 h (Figure 2). However, much more endotoxin was decomposed in the cell suspension system (from 7.4 EU/mL to 1.3 EU/mL during the initial 2 h and from 1.3 EU/mL to 0.52 EU/mL over the next 2 h), even though total quantity of the compound was greater in the latter solution than in the former. One of the main reasons is the difference in the micellar forms comparing reagent endotoxin and the endotoxin from the *E. coli* cells, because the endotoxin is an amphiphilic molecule (*23*). Another explanation has to do with small amounts of compounds added to the reagent endotoxin to stabilize the endotoxin activity. Mannitol or albumin, which are known to be hydroxyl radical (•OH) scavengers, are reported to be used as additives.

It is reported that the deactivation of endotoxin adsorbed on solid materials requires either a thermal treatment at 250 °C for more than 30 min (*24*) or a chemical treatment in ethanol containing sodium hydroxide (NaOH). However,

photocatalytic treatments can be done under ambient conditions at room temperature. Endotoxin in aqueous solution (e.g., injection, fluid) cannot be deactivated easily, usually requiring membrane filtration, adsorption by activated carbon, or affinity adsorption by polymyxin (*25*). On the other hand, the photocatalytic method is easily applied to aqueous solutions and can deactivate the endotoxin activity. The application of TiO_2 photocatalysts as antibacterial, detoxifying agents can be expected for enviromental protections, especially in medical facilities, and in pharmaceutical and medical device factories, where the endotoxin is needed to control.

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