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Journal of Photochemistry and Photobiology A: Chemistry 130 (2000) 163–170

Journal of Photochemistry and Photobiology A:Chemistry

www.elsevier.nl/locate/jphotochem

Bactericidal mode of titanium dioxide photocatalysis

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Received 7 June 1999; received in revised form 22 September 1999; accepted 23 September 1999

Abstract

When exposed to near-UV light, titanium dioxide (TiO_2) exhibits a strong bactericidal activity. However, the killing mechanism(s) underlying the TiO_2 photocatalytic reaction is not yet well understood. The aim of the present study is to investigate the cellular damage sites and their contribution to cell death. A sensitive approach using *o*-nitrophenol β-D–galactopyranosideside (ONPG) as the probe and *Escherichia coli* as model cells has been developed. This approach is used to illustrate damages to both the cell envelope and intracellular components caused by TiO_2 photocatalytic reaction. Treatment of *E. coli* with TiO_2 and near-UV light resulted in an immediate increase in permeability to small molecules such as ONPG, and the leakage of large molecules such as β-D–galactosidase after 20 min. Kinetic data showed that cell wall damage took place in less than 20 min, followed by a progressive damage of cytoplasmic membrane and intracellular components. The results from the ONPG assay correlated well with the loss of cell viability. Cell wall damage followed by cytoplasmic membrane damage leading to a direct intracellular attack has therefore been proposed as the sequence of events when microorganisms undergo TiO_2 photocatalytic attack. It has been found that smaller TiO_2 particles cause quicker intracellular damage. Evidence has been obtained that indicated that the TiO_2 photocatalytic reaction results in continued bactericidal activity after the UV illumination terminates.

Keywords: TiO_2 photocatalysis; Bactericidal mode; Permeability; ONPG assay

1. Introduction

Since the discovery of photoinduced water cleavage on titanium dioxide (TiO_2) electrodes by Fujishima and Honda in the early 1970s [1], TiO_2 photocatalysts have attracted great attention as alternative materials to aid in the purification of water and air [2–8]. TiO_2 photocatalysts generate strong oxidizing power when illuminated with UV light with wavelengths of less than 385 nm. With holes (h^+) and hydroxyl radicals ($OH^\bullet$) generated in the valence band, and electrons and superoxide ions (O_2^-) generated in the conduction band, illuminated TiO_2 photocatalysts can decompose and mineralize organic compounds by participating in a series of oxidation reactions leading to carbon dioxide. Recently, total oxidation of *Escherichia coli* cells has been demonstrated in our laboratory [9]. The reactive oxygen species (ROS) generated by the TiO_2 photocatalytic reactions cause various damages to living organisms. This is not surprising since they consist of abundant organic compounds. In 1985, Matsunaga and coworkers reported for the first time the microbiocidal effect of TiO_2 photocatalytic reactions [10]. Since then, research work on TiO_2 photocatalytic killing has been intensively conducted on a wide spectrum of organisms including viruses, bacteria, fungi, algae, and cancer cells. The first-order reaction kinetics and several modified forms have been proposed for the kinetics of bactericidal reaction of TiO_2 photocatalysts [11,12]. A recent review of this research has been published by Blake et al. [13]. Although the applications of TiO_2 photocatalyst as a microbiocide have been receiving increasing attention worldwide, the initial response of living organisms to TiO_2 photocatalytic reaction has not been studied in details and the mechanisms leading to cell death have not yet been fully understood. The studies of cell response to TiO_2 photocatalytic reactions are important to assess the fate of the cellular constituents and their effect on cell death. TiO_2-mediated photooxidations are promising for the elimination of microorganisms in many applications, e.g., self-cleaning and self-sterilizing materials. A basic understanding of the killing mechanisms would provide

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1010-6030/00/$ – see front matter
PII: S1010-6030(99)00205-1

valuable information for the optimal use of the TiO_2 photocatalyst and the rational design of TiO_2 photocatalytic reactors.

In the initial study, a decrease in intracellular coenzyme A (CoA) in the TiO_2-treated cells was detected for various microorganisms [10,14]. The direct oxidation of CoA that inhibited cell respiration and subsequently caused cell death was proposed as the first killing mode by Matsunaga et al. [10]. This mode emphasized a direct contact between TiO_2 and target cells to ensure the direct oxidation of cell components. TiO_2 photocatalytic killing studies [10,15–18] have revealed that the sensitivity of microorganisms to TiO_2 photocatalysis is likely in the following order: virus > bacterial cells > bacterial spores. This suggests that different microorganisms respond differently to TiO_2 photocatalyst due to their structural differences, particularly in the complexity and thickness of the cell envelope. However, the involvement of cell wall and cytoplasmic membrane in cell death was not taken into account until Saito and coworkers reported their transmission electron microscopy findings. They showed that the cell wall of *Streptococcus sobrinus* AHT was partially broken after cells had undergone TiO_2 photocatalytic treatment for 60 min and they recorded cell disruption after 120 min. They demonstrated that TiO_2 photocatalytic reaction induced the 'rapid' leakage of potassium ions and the 'slow' leakage of RNA and proteins. A second killing mode was therefore proposed by Saito et al.: bacterial death was caused by a significant disorder in cell permeability and the decomposition of the cell wall [15]. Sakai et al. [19] showed that treatment of cancerous cells with TiO_2 and near-UV light induced a significant increase in intracellular calcium ions. Therefore, the cell envelope may be a significant target for TiO_2 photocatlytic damage in both prokaryotic and eukaryotic cells. The intracellular macromolecules, such as nucleic acids, may be a potential target also [20]. Recently, Sunada et al. [21] demonstrated the TiO_2 photocatalytic inactivation of *E. coli* endotoxin, which is an integral constituent of the outer membrane of Gram negative bacteria. This finding suggests that the cell wall damage might take place prior to cytoplasmic membrane damage. Some investigators also reported that there was no significant difference between the time required for killing of Gram positive or Gram negative bacteria, even though the former has a thicker cell wall [22]. Nevertheless, the proposed modes did not describe the progressive damages to various components of the cell envelope and how their destruction had contributed to the overall cell killing.

In this study, we investigated the mechanisms of cell death with a focus on the gross features of cell wall and cytoplasmic membrane damages caused by TiO_2 photocatalytic reactions. We propose to establish a relationship between the stepwise cellular damages and its impact on the overall photocatalytic killing process. *E. coli* was the model organism that has been induced for the synthesis of β-D–galactosidase. The β-D–galactosidase (EC 3.2.1.23) of *E. coli* is a tetrameric enzyme with four binding sites [23]. Each identical monomer has 1023 amino acids and a molecular mass of 116,353 Da. When maximally induced, *E. coli* β-D–galactosidase accounts for up to 5% of the total cellular protein content [24]. The accessibility and fate of β-D–galactosidase upon exposure to aqueous TiO_2 illuminated with near-UV light in vitro and in vivo can scale the photocatalytic impact on both cell permeability to large molecule and enzyme inactivation. The permeability probe, *o*-nitrophenyl-β-D–galactopyranoside (ONPG, MW 301.6), is a synthetic chromogenic substrate for the intracellular β-D–galactosidase; it has been used to monitor the membrane integrity of *E. coli* [25]. During the course of TiO_2 photocatalytic treatment, the permeability of *E. coli* to ONPG was monitored by measuring its hydrolysis rate. The hydrolyzed product of ONPG, *o*-nitrophenol (ONP), gives rise to a yellow color under alkaline condition. Under normal conditions, this small hydrophilic molecule has little accessibility to the intracellular β-D–galactosidase of intact cells. The cell wall of *E. coli* imposes a minor permeability barrier, and the availability of lactose permease is the major limiting factor for the normal transport of ONPG across the cytoplasmic membrane. The substrate ONPG has to diffuse through the outer membrane and be transported across the cytoplasmic membrane in a permease-mediated process before reacting with intracellular β-D–galactosidase. Either an increase in membrane permeability to ONPG or the leakage of the enzyme to the outside of the cell can lead to the overall increase of the hydrolytic rate of ONPG. This unique property therefore can be employed as an indication for any alterations of cell wall and cytoplasmic membrane integrity. The degree of unmasking of the β-D–galactosidase activity in *E. coli* cells following TiO_2 photocatalytic reaction would indicate the extent of damages to cell structures. Furthermore, any loss of total β-D–galactosidase activity in TiO_2-treated *E. coli* cells would indicate that intracellular damage has occurred. This paper will document the process of TiO_2 photocatalytic damage and propose a new mode of cell death induced by the TiO_2 photocatalytic reaction.

It is known that TiO_2 photocatalytic killing can be significantly enhanced when sonicated TiO_2 preparation is used [26]. The enhancement may be attributed to an improvement of photoefficiency by increasing the surface area of the TiO_2 particles due to their greater dispersion. The smaller TiO_2 particles may also gain entry into cells faster and thereby promote photooxidation of critical cell components. The direct inactivation effect of sonicated TiO_2 preparation on intracellular β-D–galactosidase was also investigated.

2. Material and methods

2.1. Bacterial culture

E. coli (ATCC 27325) were grown aerobically in 250-ml conical glass flasks containing 100 ml of Luria-Bertani Broth

(Sigma, St. Louis, USA) at 32°C on a rotary shaker for 18-h. The speed of the shaker was set at 200 rpm. To provide fresh culture for the following experiments, aliquots of 0.1 ml 18-h culture was transferred to a flask containing 100 ml of Luria–Bertani Broth and 1 mM of isopropyl β-D–thiogalactopyranoside (Calbiochem, La Jolla, USA), an inducer of β-D–galactosidase synthesis, followed by incubation at 32°C for approximately 4 h with shaking. Cells were harvested by centrifuging at $4000 \times g$ for 15 min, washed twice in sterile phosphate-buffered saline (PBS, pH 7.2), and resuspended in PBS or sterile deionized water. The cell concentration was determined by a viable count procedure on Luria–Bertani agar plates after serial dilutions of the culture in PBS. To determine cell dry weight, cells were washed in deionized water and the suspension was then transferred to an aluminum weight boat in triplicates and dried at 105°C for 24 h.

2.2. Photocatalytic reaction

The photocatalyst used in this study was TiO_2 (P25, Degussa AG, Germany) with a surface area of $50\,m^2\,g^{-1}$ and a primary particle size of 20 nm. In photocatalytic experiments, stock aqueous TiO_2 suspension ($10\,mg\,ml^{-1}$ in deionized water) was always prepared immediately prior to photocatalytic reaction and kept in the dark. Washed cells (approximately $10^7\,cfu\,ml^{-1}$) were resuspended in deionized water. Aliquots of 1 ml stock aqueous TiO_2 were added to 50-ml glass beakers containing 8 ml of sterilized deionized water and 1 ml of washed cells. The TiO_2-cell slurry was placed on a magnetic stir plate with continuous stirring, and illuminated with two 40-W black-light bulbs (SYLVANIA F40/BL-B, Danvers, USA) from above. The peak wavelength of the bulbs was 356 nm. The light intensity was monitored by a long-wave UV meter (peak sensitivity 365 nm; Black-Ray®, UVP Inc., Upland, USA). The light intensity reaching the surface of TiO_2 slurry was approximately $8\,W\,m^{-2}$. The same procedure was conducted for cell lysate and pure *E. coli* β-D–galactosidase.

2.3. Cell viability assay

The loss of viability was examined by the viable count procedure. The TiO_2-cell slurry was exposed to UV light with continuous stirring. An *E. coli* suspension without TiO_2 was illuminated as a control, and the reaction of the TiO_2-cell slurry in the dark was also carried out. Samples were taken at 15-min intervals for 60 min in triplicates. The viable count was performed on Luria-Bertani agar plates after serial dilutions of the sample in PBS. All plates were incubated at 32°C for 24 h. To determine if the TiO_2 photocatalyst retains its antiseptic property long after the UV light is turned off, samples were illuminated for 30 min followed by an additional 30 min in darkness before the viable count was performed.

2.4. ONPG assay of various cell preparations

The hydrolysis of ONPG by intact whole cells was determined with washed cells in PBS. An aliquot of 9 ml washed cells was mixed with 1 ml ONPG (Calbiochem; 5 mM in PBS). Hydrolysis kinetics was determined spectrophotometrically by transferring 0.9 ml samples to cuvettes at 5-min interval for 30 min. Absorbance at 420 nm was measured by a spectrophotometer (Cary 5E, Varian Instruments, Texas, USA), after addition of 0.1 ml of a 1.0-M sodium carbonate/bicarbonate buffer (pH 10.0) to both stop the enzyme-substrate reaction and to obtain maximal absorbance. The 100% transmission value for the spectrophotometer was set using washed cells without ONPG to account for turbidity due to whole cells. The slope was determined by the linear regression of absorbance versus time. Extinction coefficient of ONP (Sigma) was determined at pH 10.0 by measuring its absorbance at 420 nm. Hydrolysis rate of ONPG was defined as released chromophore (μmole) by per mg cell dry weight per min at 20°C. Unless stated otherwise, all data were calculated from triplicate experiments.

The rate-limiting factor of ONPG transportation across the cell wall was determined using the spheroplasts of *E. coli*. The lysozyme treatment described below is a modification of the procedure described previously [27]. Washed cells were resuspended in 0.75 M sucrose (Sigma) in 10 mM Tris-HCl [2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride] (Sigma, pH 8.0). EDTA-Na_2(Sigma) was added slowly to a final concentration of 0.75 mM. The mixture was incubated at room temperature for 10 min, followed by the addition of lysozyme (Sigma) slowly to a final concentration of $20\,\mu g\,ml^{-1}$. After incubation for 5 min at room temperature, the mixture was subjected to a mild osmotic shock by a twofold dilution into deionized water to allow lysozyme penetration into the outer membrane. In order to preserve the maximal activity of β-D–galactosidase, $MgCl_2$ was added to the mixture to a final concentration of 1.0 mM. After incubation at room temperature for 5 min, 9 ml of spheroplasts was mixed with 1 ml ONPG. An ONPG hydrolytic assay was carried out as described above.

Total β-D–galactosidase activity was determined from chloroform-treated *E. coli* cells. An aliquot of 1 ml washed cells was transferred to a test tube followed by additions of 25 μl chloroform and vigorous mixing for 15 min. Under these conditions, practically maximal β-D–galactosidase activity was released. The released β-D–galactosidase was diluted 1 : 50 in PBS, and the ONPG hydrolytic assay was carried out as described above.

2.5. Changes in ONPG permeability in TiO_2-treated cells

The ONPG hydrolytic reactions were initiated by transferring 4.5 ml of illuminated and dark control TiO_2-cell slurries to the dark in duplicates, followed by the addition of an equal volume of double-strength PBS and 1 ml of ONPG.

At various intervals, TiO_2 and cells were removed by centrifuging at 4°C at 4000 × *g* for 15 min, and the pH of the supernatant was raised to 10 by the addition of a sodium carbonate/bicarbonate buffer. The absorbance of the mixture was measured by a spectrophotometer at 420 nm. The hydrolysis rates were determined as described above.

2.6. Detection of β-D–galactosidase leakage

The leakage of intracellular β-D–galactosidase induced by TiO_2 photocatalytic reaction was determined by the ONPG assay from the filtrate of TiO_2-treated cells. Sterile Acrodiscs®(pore size 0.2 μm, GelmanSciences, New York, USA) were used to collect the cell filtrate since they did not adsorb any significant amount of β-D–galactosidase while retaining all TiO_2 particles. Illuminated TiO_2-cell slurries were transferred to the dark at 10-min intervals for 90 min. Samples were kept in the dark for an additional 5 min to allow the leakage of β-D–galactosidase to build up, followed by filtering through discs using hypodermic syringes. Aliquots of 4.5 ml filtrate were transferred to a glass beaker containing 4.5 ml double-strength PBS and 1 ml ONPG. The ONPG assay of the filtrate was carried out as described above.

2.7. Detection of direct inactivation of β-D–galactosidase

The β-D–galactosidase activity in TiO_2-treated cells was determined by ONPG assay of cell lysate. The photocatalytic reaction was carried out as described above. The same experiment was conducted also with a sonicated TiO_2 suspension. The sonicated TiO_2 was prepared by sonicating the aqueous TiO_2 suspension for 5 min using Sonicator W-375 (Ultrasonics Inc., New York, USA) fitted with a microtip and set at 50% output power. Aliquots of 1 ml TiO_2-cell slurry illuminated for 10 to 90 min were then subjected to the chloroform treatment as described before. Cell lysates were diluted 1 : 10 in PBS. Aliquots of 9 ml diluted cell lysates were mixed with 1 ml ONPG. The ONPG assay was carried out as described above.

The effect of TiO_2 photocatalyst on cell-free β-D–galactosidase was examined using cell lysate. An aliquot of 1 ml washed cells was subjected to the chloroform treatment as described above. The released β-D–galactosidase was diluted 1 : 10 in deionized water and subjected to TiO_2 photocatalytic treatment as described before. The illuminated cell lysates were transferred to the dark at 10 min intervals for 30 min followed by the ONPG assay as described above.

The effect of TiO_2 photocatalyst on pure β-D–galactosidase was also examined using the ONPG assay. *E. coli* β-D–galactosidase (approximately 50 μg ml^{-1}; G-2513, Sigma) was mixed with TiO_2 (0.1%) and subjected to near-UV illumination. The illuminated TiO_2-enzyme mixtures were transferred to the dark at intervals of 1, 2, 5, 10, 20, 30, and 60 min. ONPG hydrolytic reactions were then initiated by the addition of ONPG to the mixture and incubated for 5 min. TiO_2 was removed by centrifugation at 4°C and the pH of the supernatant was raised to 10 by the addition of a sodium carbonate/bicarbonate buffer. The absorbance of the supernatant was measured by a spectrophotometer at 420 nm.

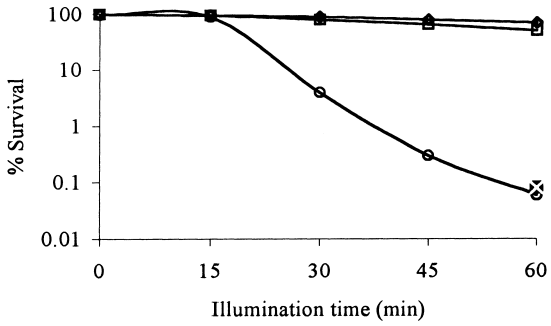

Fig. 1. The effect of TiO_2 photocatalytic reaction on cell viability. The survival curves were obtained from the viable count of TiO_2 (1.0 mg ml^{-1}) and/or UV light (8 W m^{-2}) treated *E. coli* cells. The initial cell concentration was 10^6 cfu ml^{-1}. (◇), cell +TiO_2 in dark; (□), cell +light; (○), cell +TiO_2 +light; (×), cell +TiO_2, 30 min in light and 30 min in dark.

3. Results

3.1. Loss of viability under TiO_2 photocatalytic reaction

The viability of TiO_2-treated cells was determined by colony counting after 24 h of incubation. The survival curve in Fig. 1 shows that when *E. coli* cells (approximately 10^6 cfu ml^{-1}) underwent illumination for 15 min in the presence of 1 mg ml^{-1} TiO_2, almost all of the cells were still viable. After 20 min of treatment, however, only 12% of the cells retained their viability. At the end of 30 min of illumination, more than 96% of the cells lost their viability. Complete killing was achieved after 60 min of illumination. It was observed that if the light was turned off after 30 min followed by an additional 30-min incubation in darkness, the viable cell count obtained at 60 min was similar to the sample that had undergone illumination continuously for 60 min (Fig. 1).

3.2. Change in ONPG permeability during TiO_2 photocatalytic treatment

The effect of TiO_2 photocatalytic reaction on cell permeability was examined using the ONPG assay. ONPG assays were performed for intact whole cells, spheroplasts, and lysed cells. The baseline levels of the ONPG hydrolysis rates obtained from these preparations demonstrate the effect of various levels of cell wall and cytoplasmic membrane removal. Under normal conditions, the outer membrane and the cytoplasmic membrane of *E. coli* cells have very limited permeability for ONPG. The low accessibility of ONPG to

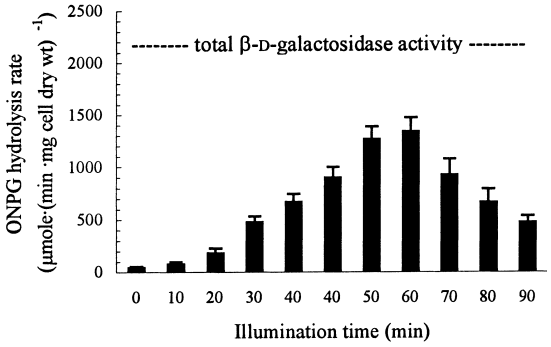

Fig. 2. Effect of TiO_2 photocatalytic reaction on cell permeability. TiO_2-*E. coli* slurry containing 10^6 mg ml^{-1} cells and 1.0 mg ml^{-1} TiO_2 was illuminated with UV light (8 W m^{-2}). ONPG assay was initiated by mixing TiO_2-treated cells with 0.1 mM ONPG. TiO_2-mediated membrane damage was shown by the changes in ONPG hydrolysis rate. Total β-D–galactosidase activity (dashed line) represents the total accessibility of β-D–galactosidase in untreated *E. coli* cells.

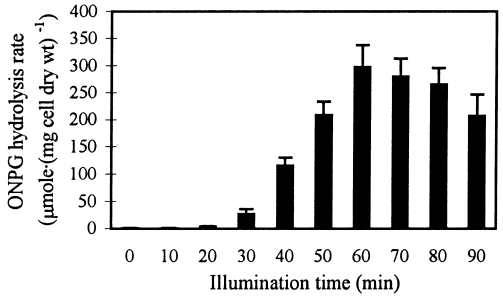

Fig. 3. The leakage of intracellular β-D–galactosidase induced by TiO_2 photocatalytic reaction. Cell filtrates were obtained from cells treated with TiO_2 and UV light. The presence of β-D–galactosidase in the cell filtrate was determined by ONPG assay, and β-D–galactosidase activity was expressed as the ONPG hydrolysis rate.

the β-D–galactosidase in intact cells is reflected by a hydrolysis rate that is less than 2% (43.8 ± 10.2 μmol min^{-1} mg cell per dry wt) that of the total activity measured for the lysed cells (2218.5 ± 41.5 μmol min^{-1} mg cell per dry wt). To quantify the permeability barrier imposed by cell wall, *E. coli* spheroplasts devoid of the outer membrane and peptidoglycan layers were prepared. As expected, a slight increase in ONPG hydrolytic rate was observed with the spheroplast preparation (9.5% of the maximum, 210.3 ± 41.5 μmol min^{-1} mg cell per dry wt) where the cytoplasmic membrane was still intact. Any further increase in hydrolytic rates beyond the 9.5% level would indicate that the intact structure of the cytoplasmic membrane is compromised. The ONPG assay of TiO_2-treated cells therefore can be used to gauge the degree of cell wall and cytoplasmic membrane damage and assess any correlation of that with cell death.

Fig. 2 shows the kinetics of TiO_2 photocatalytic reaction on the destruction of the cell semipermeability. A slight increase in ONPG accessibility was observed after 5 min of illumination, albeit at a slow rate. This indicates initial damage to the cell wall. The barrier posed by outer membrane and peptidoglycan layers was partially removed between 10 and 20 min of reaction, as their hydrolytic rate exceeded the 9.5% benchmark imposed by the cell wall. Between 20 and 50 min the rate of increase in ONPG hydrolysis was linear with illumination time and reached a maximum at 60 min. The result implies that once the protection provided by the outer membrane and peptidoglycans layers was removed, the cytoplasmic membrane damage took place at a much faster rate. Kinetic data on loss of selective permeability in Fig. 2 coincided well with the kinetics of cell viability from Fig. 1. Permeability data implied that when only the cell wall was damaged, no loss in viability was detected. Damages to the cell wall can be repaired during the subculture of cells onto agar plates for the viability study [28]. However, a more severe impact on survival was observed between 15 and 20 min of treatment. This occurrence parallels the onset of cytoplasmic membrane damage that is more deadly and irreversible. The maximal hydrolysis rate of ONPG, i.e., 60% of the maximum, was obtained at 60 min. After 60 min, the rate of ONPG hydrolysis declines, strongly suggesting a loss of enzyme activity.

Data in Fig. 2 provide insight into the increase in permeability to a small molecule such as ONPG. To determine if TiO_2 photocatalytic reaction had resulted in any leakage of larger molecules, β-D–galactosidase was used as a marker. The leakage of the enzyme from the TiO_2-treated cells to the extracellular fluids was measured by the ONPG assay. The β-D–galactosidase activity in the filtrate recovered after illumination of TiO_2-cell slurry was determined. The filtrates were collected from TiO_2-treated cells after different periods of illumination. Each sample was allowed to stand an additional 5 min in the dark to allow the leakage of β-D–galactosidase to build up. The presence of β-D–galactosidase in the cell filtrate was detected after 20-min illumination of the TiO_2-cell slurry (Fig. 3). Between 30 and 60 min, the leakage of β-D–galactosidase increased at a linear rate. After 60 min of illumination, approximately 13% of the total intracellular β-D–galactosidase had leaked to the outside of the cells. Further illumination resulted in a slight decrease of β-D–galactosidase activity in the extracellular fluid, presumably due to the deactivation of the enzyme during the photocatalytic treatment.

3.3. Effect of TiO_2 photocatalytic reaction on intracellular components

ONPG assay of TiO_2-treated cells consistently showed a decrease in the β-D–galactosidase activity as measured by the hydrolytic rate of ONPG after 60-min of illumination (see Fig. 2). Similar results were also obtained using cell filtrate (see Fig. 3). Together, these data suggest a decline of the amount of active β-D–galactosidase both in the whole cell and in the extracellular fraction after long-term TiO_2 treatment (>60 min), probably due to photocatalytic alterations of the enzyme itself. To verify that illuminated

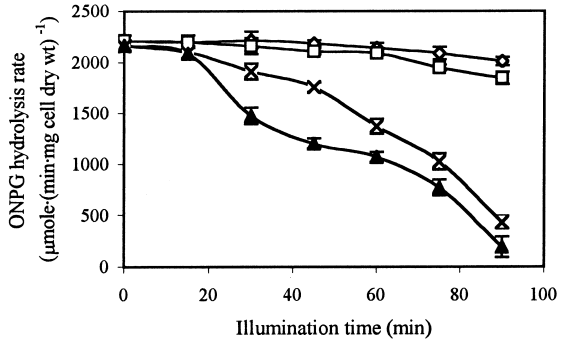

Fig. 4. Direct effect of TiO_2 photocatalytic reaction on the intracellular β-D–galactosidase activity. TiO_2-treated cells were lysed by chloroform treatment, and the remaining β-D–galactosidase activity was determined by ONPG assay. The loss of β-D–galactosidase activity was compared for both unsonicated- and sonicated-TiO_2slurry. (◇), cell +TiO_2 in dark; (□), cell +light; (×), cell +TiO_2 +light; (▲), cell +sonicated-TiO_2 +light.

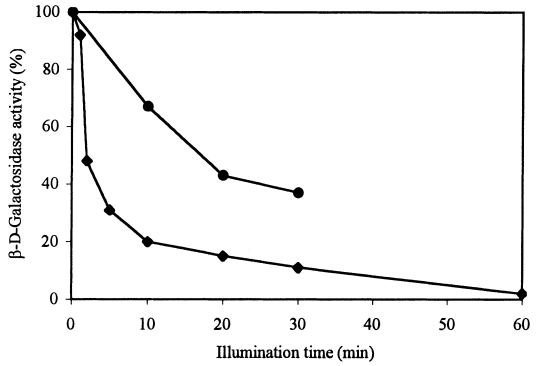

Fig. 5. Effect of TiO_2 photocatalytic reaction on activities of cell-free β-D–galactosidase and pure β-D–galactosidase. Cell-free extract was obtained from chloroform-treated *E. coli* cells (10^5 cfu ml^{-1}). Cell−free extract and pure β-D–galactosidase (∼50 mg ml^{-1}) were mixed with TiO_2 slurry (1.0 mg ml^{-1}) and exposed to near-UV light for various times. β-D–Galactosidase activity of the TiO_2-treated cell extract was measured by ONPG assay and expressed as the percentage of control. TiO_2 photocatalytic reaction showed significant inactivation effect on the total β-D–galactosidase in the extract (●) and pure β-D–galactosidase (◆).

TiO_2 particles also had a deleterious impact on intracellular component directly, the effect of TiO_2 photocatalytic reaction on the intracellular β-D–galactosidase was studied. Cells were treated with illuminated TiO_2 prior to lysis with chloroform. Results are shown in Fig. 4. A decline in β-D–galactosidase activity following TiO_2 photocatalytic reaction was indeed detected after 30 min of illumination. A faster decrease in residual β-D–galactosidase activity was seen initially for the sonicated TiO_2. However, the decline in rate of β-D–galactosidase activity became the same for both sonicated and unsonicated TiO_2 after 60 min of illumination. This suggests that the sonicated TiO_2, which is likely to contain a larger fraction of submicron particles compared to the unsonicated preparation (data not shown), can attack intracellular components more effectively at the early stages of the photocatalytic attack when semipermeability is not fully compromised.

3.4. Direct inactivation of β-D–*galactosidase in vitro*

To demonstrate that TiO_2 photocatalytic reaction is effective in inactivating the β-D–galactosidase even in the presence of whole cell organic matter, ONPG assay was performed for cell lysate that had undergone TiO_2-photocatalytic reaction. Fig. 5 shows a linear decline of cell-free β-D–galactosidase activity upon TiO_2 photocatalytic reaction. Light alone, or TiO_2 in the dark, had negligible effect on the cell-free β-D–galactosidase. This verifies that illumination in the presence of TiO_2 resulted in a general decrease of β-D–galactosidase activity regardless of its location. When pure β-D–galactosidase enzyme was in contact with TiO_2 under near-UV light illumination, the loss of enzyme activity was detected immediately at the onset of the photocatalytic reaction (see Fig. 5). TiO_2 photocatalysts exhibit a very strong inactivation effect on this enzyme in vitro.

4. Discussion

In the present study, a sensitive approach using the ONPG assay has been adapted successfully to examine TiO_2-mediated damages on various cellular sites and their contribution to cell death. The unique advantage of using the ONPG assay for TiO_2-treated cells is that this rapid assay can provide signals to distinguish various cellular damage processes and the location of such occurrence more precisely. The killing kinetics obtained in this study confirms earlier findings that illuminated TiO_2 exerts strong biocidal actions toward *E. coli* [11,29–33]. Evidence for increased cell permeability during photocatalytic treatment from the ONPG assay (Fig. 2) correlates well with the loss of cell viability (Fig. 1). Nevertheless, the ONPG assay has some limitations. For example, the assay is only suitable for those microorganisms that possess β-D–galactosidase, and the assay is not able to determine the chemical details underlying the process of oxidative damage.

ONPG assay of TiO_2-treated cells showed that photocatalytic reaction not only causes a permeability increase to both ONPG (influx) and β-D–galactosidase (efflux) but can also inactivate intracellular β-D–galactosidase. Kinetic data demonstrate that the cell wall damage takes place immediately after TiO_2treatment and allows small molecule such as ONPG to diffuse through the outer membrane more rapidly (Fig. 2). The barrier imposed by the outer membrane and peptidoglycan layer was severely destroyed by the photocatalytic reaction between 10 and 20 min. This was followed by a progressive damage of the cytoplasmic membrane. At this stage, the cell becomes permeable even to large molecules such as β-D–galactosidase, as evidenced by the significant amount of β-D-galactosidase recovered from the filtrate after 30 min of TiO_2 photocatalytic reaction (Fig. 3). At this

stage, cytoplasmic membrane has been destroyed severely, which explained the irreversible loss of viability shown in Fig. 1. This confirms that the cell envelope of microorganisms is indeed the initial target of TiO_2 photocatalytic reaction and a primary cause of cell death.

Long-term photocatalytic reaction causes a decline of intracellular β-D–galactosidase activity. The unique biphasic kinetics in Fig. 2 clearly indicates that TiO_2 photocatalytic reaction has severe impact on both the cell envelope and intracellular constituents. The loss of enzymatic activity was also observed for cell filtrate and cell lysate (see Figs. 3 and 4), suggesting that the photocatalytic reaction of TiO_2particles can cause significant damage to intracellular components, such as the alterations of protein structure. The effect of TiO_2 photocatalysts on β-D–galactosidase in vitro has been shown in Fig. 5, using both a cell-free extract and a pure enzyme, respectively. Data from Fig. 5 show that TiO_2 photocatalytic reaction can effectively inactivate β-D–galactosidase even in the presence of whole cell organic matter. Recently, Sakai and coworkers [19] have reported that treatment of eukaryotic cells with TiO_2 and UV light caused a significant increase in intracellular calcium content because of an increase in cell permeability to the extracellular Ca^{2+}. The uptake of TiO_2 particles in eukaryotic cells may result from phagocytosis [34]. Once TiO_2 particles gain entry into cells (in this case, after cytoplasmic membrane is damaged), intracellular components might be exposed to a direct attack. Cell constituents such as amino acids and nucleic acids can be photodegraded in vitro, when exposed to UV light in the presence of TiO_2 [20,35,36]. Evidence appears to support that TiO_2 photocatalyst has no significant selectivity in attacking cellular components. Initial oxidative damages were determined by the location of the TiO_2 particles and the migration distance of radicals. Damage to intracellular content can be effectively prevented initially by the cell wall and cytoplasmic membrane. This explains why whole cells (10^6 cfu ml^{-1}) can maintain certain enzymatic activity and cell viability after 30-min illumination in the presence of TiO_2(1 mg ml^{-1}). However, when the integrity of the cell wall and subsequently cytoplasmic membrane are compromised by the TiO_2 photocatalyst, cellular constituents then become the direct targets of photocatalytic TiO_2 attack, either by means of the efflux of cellular contents or the influx of TiO_2 particles. This proposed mechanism emphasizes the importance of the cell wall and cytoplasmic membrane as the primary targets of TiO_2 attack. This is particularly important for photocatalytic reactors with immobilized TiO_2. In this case, cell permeant radicals, e.g., H_2O_2, may play crucial roles in cell death. Once the integrity of the cell envelope becomes compromised, intracellular components begin to leak from the cell and free TiO_2 particles may also diffuse into the damaged cells and directly attack the secondary targets. Once inside the cell, the targets of photocatalytic attack can include enzymes [10,14] and DNA [20]. This may be true also for ultrafine TiO_2 particles in a slurry phase.

It has been shown that sonicated TiO_2 preparation was more effective in causing intracellular damage (see Fig. 4). The killing rate can also be significantly enhanced when sonicated TiO_2 preparation was used [12]. The enhancement may be attributed to an improvement of photoefficiency by increasing the surface area and, hence, the dispersion of TiO_2 particles. The smaller TiO_2 particles may also gain entry into cells faster and thereby promote direct photooxidation of critical cell components. Sonolysis of TiO_2-cell slurry during near-UV illumination also enhances the microorganism inactivation, probably due to cell wall rupture and mass transport increase [12].

Pham et al. [18] has reported that intermittent illumination reduced viable *Bacillus pumilus* spores more effectively than continuous exposure to UV. Whether this can be considered a form of residual disinfection capacity for photodisinfection using TiO_2 is open to discussion [37]. In the present study, it has been demonstrated that TiO_2-treated cells continue to lose their viability even after UV light is turned off. Data from Fig. 1 suggest that TiO_2 particles remaining in the slurry may still retain their bactericidal activity. Another explanation is that certain lethal reactions would continue to propagate even after the UV illumination stops. This effect may be masked by the standard viable count procedure involving serial dilutions in buffered saline, as it allows cells to form colonies on rich nutrient media, which in turn allows the injured cell to recover. Nevertheless, once lethal oxidation reactions are initiated by the TiO_2 photocatalytic reaction in complex cellular systems, the damage may continue in the dark via the Fenton reaction or the free radical chain reactions of lipid peroxidation [38]. It is not surprising that cells would lose viability even after the removal of TiO_2 and/or UV light.

5. Conclusion

From currently available evidence and this work, we propose a more detailed mechanism for the bactericidal effect of TiO_2 photocatalytic reaction. The initial oxidative damage takes place on the cell wall, where the TiO_2 photocatalytic surface makes first contact with intact cells. Cells with damaged cell wall are still viable. After eliminating the protection of the cell wall, the oxidative damage takes place on the underlying cytoplasmic membrane. Photocatalytic action progressively increases the cell permeability, and subsequently allows the free efflux of intracellular contents that eventually leads to cell death. Free TiO_2 particles may also gain access into membrane-damaged cells, and the subsequent direct attack on the intracellular components can accelerate cell death. Evidence for the molecular targets of attack in the cell envelope is the subject of another report from our laboratory in which we demonstrated that lipid peroxidation can be initiated via TiO_2 photocatalytic reaction, and may be an important cause of cell death [38].

Acknowledgements

This study was supported by the FIRST program at the National Renewable Energy Laboratory and the Center for Indoor Air Research.

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