TEM evidence of ultrastructural alteration on Pseudomonas aeruginosa by photocatalytic TiO_2 thin films

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Abstract

The antibacterial efficiency of longwave UV-irradiated TiO_2 thin films as well as the ultrastructural damage on bacterial cells was evaluated using Pseudomonas aeruginosa as a model. The quantitative antibacterial efficiency assays showed a bacterial inhibition in the range of 32–72% at different times of irradiation. Transmission electron microscopy (TEM) was used to detect the effect of irradiation of TiO_2 thin films on the ultrastructure of the bacterial cell in order to reveal possible cellular damage. After 40 min irradiation, an abnormal cellular division was observed: instead of a normal septum, an ‘elongated bridge’ was formed. At a longer irradiation time, wavy structures all around the outer cell membrane were observed, and also some bubble-like protuberances, which expelled inner material. The mechanism of irreversible bacterial cell damage caused by the photocatalytic effect of TiO_2 could be related to abnormal cell division, aside from the reported physicochemical alteration of the cell membrane.

Keywords: Titanium dioxide, Photodegradation, Antibacterial effect, Bacterial inhibition, Photocatalysis.

Introduction

The discovery of the electrochemical photolysis of water with a TiO_2 electrode [1] has triggered research on the properties of this material [2]. In 1985, Matsunaga et al. [3] first reported the antibacterial effect of TiO_2 photocatalytic action: bacteria cultures in

contact with UV- irradiated TiO_2- Pt thin film, during 60 to 120 min, had a significant reduction in number of cultivable cells. Since this report, the photocatalytic property has been widely studied in a variety of microorganisms such as viruses, bacteria, fungi, algae, and also in cancer cells [4,5]. However, the lethal mechanism of TiO_2 is not well understood yet. Some authors have proposed that the cell wall is the target of this effect. Sunada et al. [6] showed endotoxin degradation in E. coli, and Pinching et al [7] suggested the peroxidation of lipids as the initial effect. Few papers deal with the structural damage on the bacterial cell; Saito et al- [8] reported by TEM analysis a complete destruction of Streptococcus sobrinus AHT cells, after 60-120 min of photocatalytic action. They suggested changes in cell membrane permeability.

In previous work, pure and doped UV-irradiated TiO_2 thin films were tested on their antibacterial effect and no significant differences among them were found [9]. Therefore, using pure UV-irradiated TiO_2 thin films, the investigation on the mechanism of photocatalytic action over the bacterial cell was undertaken. In this work, we report the preparation and bactericidal effect of TiO_2 thin films deposited on soda lime glass slides by sol-gel technique [10]. Furthermore, TEM analysis on the ultrastructural damage to Ps. Aeruginosa cells caused by UV-irradiated TiO_2 thin films is reported.

Materials and methods

Thin film preparation and characterization: Sol-gel thin films were obtained by a similar method to that reported in the literature [10]. The precursor solution postwas prepared by adding drop-wise, 1.5 ml of $TiCl_4$ in 15 ml of ethanol at room temperature. A large amount of HCl gas was exhausted during the mixing process. A TiO_2 precursor film was deposited (3–4 drops) onto the glass slide using the spin-coating method,

conducted at 8500 rev. /min for 30 s. After that the film was dried at room temperature, and placed for sintering in a cylindrical furnace at 450 °C for 6 h. The furnace temperature was raised at 4 °C/min.

Sol–gel samples were analyzed by X-ray diffraction (XRD) to determine the crystalline phase of the film. Spectral reflectance measurement in the UV–Vis interval was also performed to determine the thickness of the films. Table 1 summarizes the optimized experimental conditions rate, crystalline phase) are summarized in Table 1. The used in the preparation of both types of films.

Quantitative inhibitory test: A wild type Ps. aeruginosa was used in the inhibitory tests (Facultad de Ciencias Químicas-UACH Strain Collection). Bacteria were cultured on Brain-Heart broth for 4 h at 37 °C and 250 rev. /min. A bacterial inoculum (0.15 cm^3), with known initial cell concentration (10^8 colony forming units (CFU cm^{-3})) was placed on top of the film-covered substrates and on a glass slide without any film as control. To limit and fix the contact area between the inoculum and the film, a Teflon ring was firmly attached to the glasses; this ring also served to establish the height of the inoculum volume over the film's surface [9]. The film-covered substrates and control were placed inside an irradiation chamber, and exposed to a long-wave UV light (365 nm) for 40, 80, 120 and 240 min. The irradiance of the lamp at 365 nm was measured by means of a radiometer UVP model UVX. After exposure, 1 ml of the bacterial inoculum was recollected from the Teflon ring, to determine the final cell viability [9]. Serial dilutions to a final concentration of 10^{-7} and spread plate techniques on Plate Count medium were used to determine initial and final bacterial cell numbers (CFU) [11].

Observation of TiO_2 thin film effect by TEM: After irradiation, 1 ml of the known inoculum was centrifuged and fixed by 2.5% glutaraldehyde in 0.1 mol dm^{-3} cacodylate buffer for 2 h, washed three times with 0.1 mol dm^{-3} cacodylate buffer (pH 7.2–7.4) and postfixed for 1 h in 1% osmium tetraoxide. The specimens were dehydrated by a graded series of ethanol (75, 85, 95, and 100%) and embedded in Epon 812. Ultrathin sections were obtained, contrasted with uranyl acetate and lead citrate and observed with a JEOL 1010 transmission electron microscope.

Table 1
Experimental conditions used in the preparation of TiO_2 thin film on glass slides by sol–gel process

Film	Material	T_s (°C)	t (min)	d (nm)	Phase TEM$^+$–XRD
TiO_2-1	TiO_2	450	360	98	Anatase
TiO_2-2	TiO_2	450	360	192	Anatase

Material type of film; T_s, sintering temperature; t, sintering time; d, film thickness; phase, crystalline phase of the films.

Results

TiO_2 thin film characteristics: The characteristics of TiO_2 thin films (thickness, growth rate, crystalline phase) are summarized in Table 1. The films were transparent and non-light scattering as deduced from characterization analysis reported elsewhere [12]. XRD spectra of TiO_2 films showed that the diffraction peaks had a very low intensity indicating that the material was poorly crystalline. Despite this, the observed peaks corresponded to the anatase phase (Table 1).

Antibacterial activity: In order to estimate the antimicrobial effect of irradiated TiO_2 thin films on Ps. aeruginosa, a quantitative assay was performed. Fig. 1 shows the

results of this test. At 40 min, a marked reduction on the number of cells was observed, with inhibition ranging from 60 to 72%. However, at 80 min percentages of inhibition ranged from 38 to 49%. At longer inhibition times, inhibition was around 32% at 120 min and 59–69% at 240 min.

Observation of film effect by TEM: In order to identify the probable damage mechanism

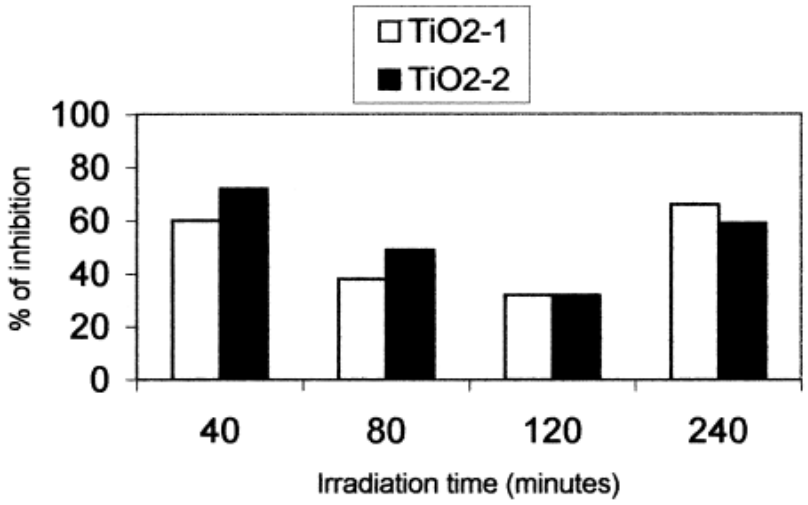

Fig. 1. Antibacterial response of titanium dioxide thin films. At 40 min, a marked reduction on the number of cells was observed, with inhibitions ranging from 60 to 72%. At 80 min there was an increase in cell population, and percentages of inhibition ranged from 38 to 49%. The same films were evaluated with longer treatment time, and inhibitions were 32% at 120 min and 59–69% at 240 min.

caused by long-UV irradiation of bacteria in contact with the TiO_2 thin films, the irradiated cells were processed for TEM analysis.

Firstly, at low magnifications we were not able to observe major changes. At high magnifications we observed several features.

(a) Control bacteria. That is, bacteria that have not been in contact with the thin film, or have not been irradiated, had a well-preserved ultrastructure. The cell wall is

typical: thick, rounded and uniform (Fig. 2a, ar-rowheads). The inner material is also normal. Several samples presented clear spots (Fig. 2a, arrows). A longitudinal view of the bacterial cell showed good preservation of the cell wall and inner material (Fig. 2b). No change was observed even at long periods (240 min), when bacteria were irradiated without TiO_2 thin film. No control cell showed any ultrastructure feature indicating damage.

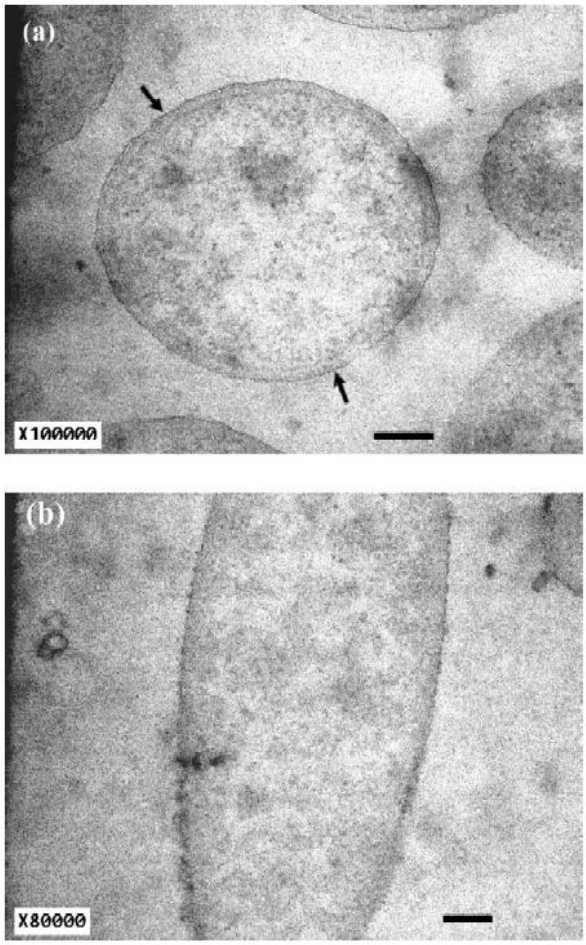

Fig. 2. *Ps. aeruginosa* control cells. (a) Cross-section of *Ps. aeruginosa* without any treatment showing typical morphology: uniform and rounded. The cell envelope shows normal thickness and shape (arrows). Bar=100 nm. (b) Longitudinal section of bacterial cell; 240 min incubation time with irradiation only. The cell showed normal morphology. Bar=200 nm.

(b) Longwave UV-irradiated bacteria in contact with TiO_2 thin films for 40 min presented abnormal features concerning its cell division, in contrast to the control bacteria that did not show these features. The normal cell division forms a septum between two cells. This septum grows until the whole cells separate, and they may remain joined for a short while, forming long chains of bacteria. By the time of cell division, the irradiated bacteria in the presence of TiO_2 thin films did not form a septum. Instead, they develop an incomplete cell separation with a 'bridge' between the daughter cells (Fig. 3a, arrows); this feature can be observed even at low magnifications. Deformed daughter cells were observed with the 'bridge' between them (Fig. 3b). Many bacteria showed an enlarged 'bridge' (Fig. 3c). The cell wall is easily noted in this structure (Fig. 3c, arrows), and was well preserved. No cell blasting was observed.

(c) At longer irradiation time (240 min) the bacteria showed another aspect of damage. The most prominent of such damage was the loss of the characteristic shape of the cell envelope: from typically rounded and uniform to abnormally wavy and blasted (Fig. 4). Many sections of the cell envelope were thin (Fig. 4a, arrows) resembling an indented wheel. The inner material was apparently homogeneous. The following most frequent damage was cell enlargement, with portions of putative daughter cell (Fig. 4b, arrow). Furthermore, many of the bacteria showed cell blasting in different stages. Some bacteria showed small protuberances, probably containing cell inner material, without a visible envelope but the remains of the latter were very close (Fig. 4c, arrows). Many bacteria presented abnormalities (Fig. 4d): non-homogeneous cell envelope but plenty of protuberances (Fig. 4d, thin arrows). Two big protuberances

were surrounded by a cell envelope (Fig. 4d, arrowendings). Little cell materials were present in these protuberances (Fig. 4d, arrowheads).

Discussion

The antibacterial effect of TiO_2 thin films is associated to their photocatalytic property, and directly related to their thickness. Films used in this work were about 100 nm thick (Table 1) which has been reported to be highly photocatalytic [13]. Results of percentage of bacterial inhibition obtained in this work are similar to those reported in the literature [5,7–9,14–18]. However, as it can be observed in Fig. 1, there was a recurring effect in the percentage of inhibition with respect to irradiation time. The duplication time of the Ps. aeruginosa strain used in this experiment, under the conditions tested, was around 50 min. The recurring effect could be explained by the competition of the growth of bacterial cell population and the destruction of those that were lethally affected by the irradiated TiO_2. At 40 min, the original population inoculated decrease in number, due to the destruction of those irradiated bacteria on TiO_2 thin films, since at this stage the original population did not duplicate, yet. However, at 80 and 120 min many of the non-lethally affected cells could duplicate, and the percentage of inhibition went down. Finally, at 240 min, the growth rate of bacterial cell population diminished and again the percentage of inhibition increased. Therefore, due to the high bacterial concentration used in this work, the antibacterial effect of the TiO_2 photocatalyst was masked with the growth of those bacterial cells that were not lethally affected by the irradiated TiO_2 thin film.

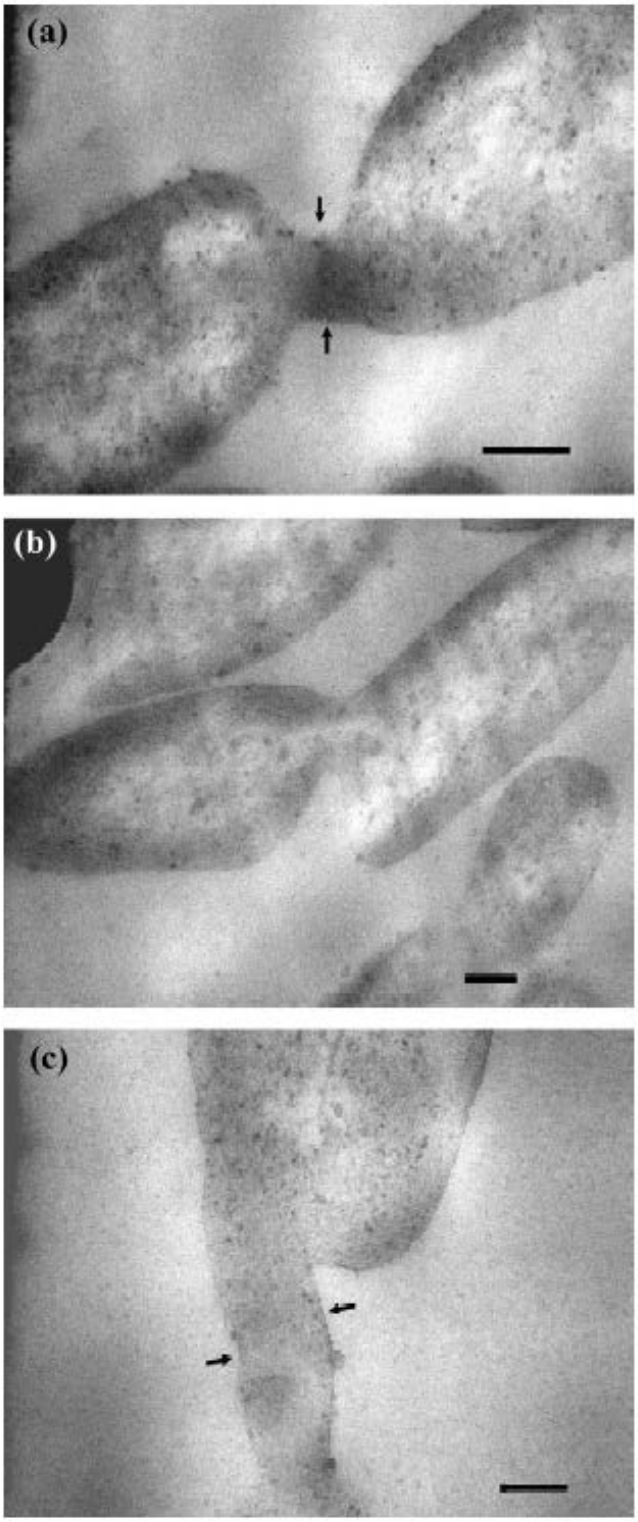

Fig. 3. *Ps. aeruginosa* cells treated for 40 min. Bacterial cells were placed in TiO_2 thin films and irradiated for 40 min. (a) Two dividing cells showed, instead of division septum, a 'bridge' (arrows). (b) Abnormal cell division with loss of typical morphology. (c) 'Bridge' elongation (arrows) of cells with abnormal cell division. There is a continuum of cell wall on the 'bridge'. The inner material remains morphologically normal. Bar= 200 nm.

However, the lethal mechanism is not well known. Few studies have been reported in regards to this mechanism [3,4,7,8], and the authors concluded that the conjunction of TiO_2 and irradiation produced damage on the cell wall. The timely serial experiments performed in this work were done to gather evidence about the mechanism of inhibition. It can be pointed out that: (a) by the time the bacteria divide abnormal cell separation was observed (Fig. 3). Instead of forming a septum which eventually separates the dividing cells, we observed the formation of a 'bridge' between dividing cells, resembling the cytokinesis in eukaryotic cells; (b) at longer time the bacterial cell had abnormally irregular-shaped wall; furthermore, many cells showed vesicles which slough off the cell. In contrast, the control cells had a uniform wall and did not have this kind of vesicles. One of the possible causes of the latter was the presence of discontinuities of the cell wall with the exit of intracellular material. However, the presence of material that resembles the cell wall around these vesicles led us to consider a possible abortive division. Nevertheless, the TEM micrographs of the irradiated bacteria in the presence of TiO_2 film observed were like the bacterial damage reported by the effect of aminoglycoside antibiotics on the Ps. aeruginosa outer membrane [19]. Similar micrographs were presented recently by Belaaouaj et al. [20], which also showed the degradation of protein A in E. coli which is on the outer membrane.

Conclusions

Due to the high bacterial concentration (~10^8 CFU ml $^{-1}$) used in this work, the antibacterial effect of the TiO_2 thin films was masked with the growth of those bacterial cells that were not lethally affected by the photocatalytic effect of the irradiated TiO_2 thin film.

In regard to the mechanism of inhibition, the gathered data led us to propose that it might be biochemical damage related to the cell division process. It might be possible that the bacterial cell had multiple sites of cell division, because the irradiation of the bacteria in the presence of TiO_2 thin films triggered this mechanism on several sites. Another possibility was cell wall weakening, either by a weak layer of peptidoglycan or by abnormal lipids on the cell wall. The multiple sites of cell division is a novel point of view, because earlier reports deal exclusively with cell wall damage.

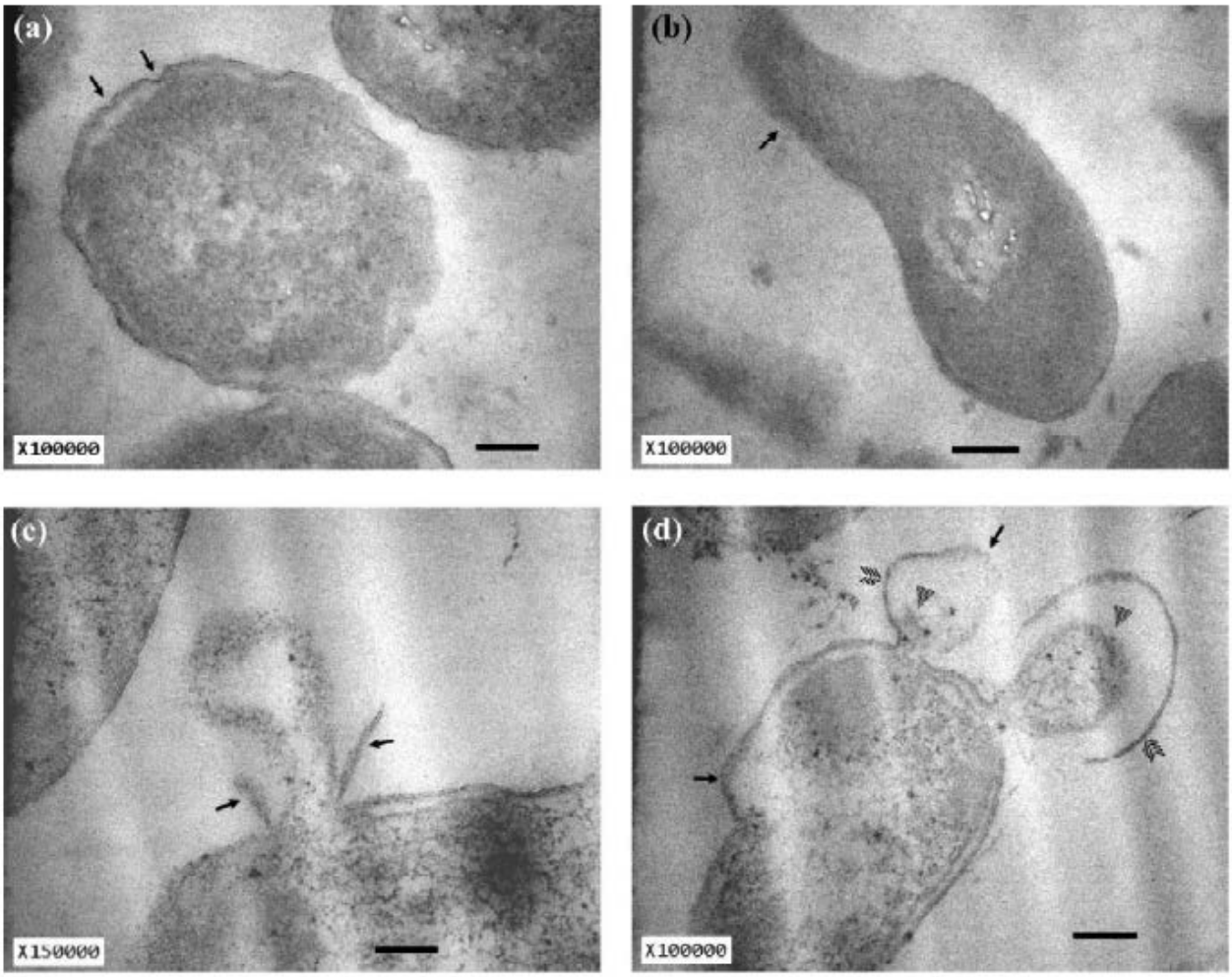

Fig. 4. *Ps. aeruginosa* cells treated for 240 min. Bacterial cells were placed in TiO_2 thin films and irradiated for 240 min. (a) The cell showed abnormal wavy cell walls (arrows). The inner material looked apparently normal. (b) Bacterial cell with loss of longitudinal shape. There is a protuberance (arrow), probably a beginning of abnormal cell division. (c) 'Bubble' formation on bacterial cell wall. This protuberance had a cell wall presence (arrows). (d) Multiple bubbles on cell wall. Earlier bubbles (thin arrows). Two big bubbles were surrounded by a cell envelope (Fig. 4d, arrowendings). Few cell materials were present in these protuberances (Fig. 4d, arrowheads). Bar=200 nm.

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