



The bactericidal effect of TiO₂ photocatalysis involves adsorption onto catalyst and the loss of membrane integrity

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Introduction

Studies in the past few years have revealed that classical disinfection by chlorine or ozonation can generate carcinogenic and mutagenic by-products (DBPs), thus boosting research on alternative methods, such as photocatalysis (Bellar *et al.*, 1974; Rook, 1974; Nieuwenhuijsen *et al.*, 2000). This process is based on the ability of a semi-conductive catalyst (TiO₂) to kill bacteria upon illumination in aqueous solution (Watts *et al.*, 1995; Herrera Melian *et al.*, 2000; Arana *et al.*, 2002; Dunlop *et al.*, 2002; Ibanez *et al.*, 2003). Several studies have addressed issues such as disinfection kinetics, TiO₂ concentration, light intensity, dissolved salt concentration and pH (Sun *et al.*, 2003; Rincon *et al.*, 2004), but the basis for the bactericidal effect of photocatalysis is not well established.

Active TiO₂ in anatase crystalline form behaves as a classical semi-conductor. Illumination of TiO₂ in water with light below 385 nm generates electrons in the conduction band (e_{cb}^-) and positive 'holes' in the valence band (h_{vb}^+). In aqueous environments, electrons (e_{cb}^-) react with O₂ and holes (h_{vb}^+) with HO⁻ or H₂O to form reactive oxygen species (ROS). Efficient photocatalysis requires the presence of a strong electron acceptor, like O₂ or, better, H₂O₂ to prevent recombination of e_{cb}^- and h_{vb}^+ (Wei *et al.*, 1994). Hence, the bactericidal effect of photocatalysis with TiO₂

Abstract

The bactericidal effect of photocatalysis with TiO₂ is well recognized, although its mode of action is still poorly characterized. It may involve oxidation, as illuminated TiO₂ generates reactive oxygen species. Here we analyze the bactericidal effect of illuminated TiO₂ in NaCl–KCl or sodium phosphate solutions. We found that adsorption of bacteria on the catalyst occurred immediately in NaCl–KCl solution, whereas it was delayed in the sodium phosphate solution. We also show that the rate of adsorption of cells onto TiO₂ is positively correlated with its bactericidal effect. Importantly, adsorption was consistently associated with a reduction or loss of bacterial membrane integrity, as revealed by flow cytometry. Our work suggests that adsorption of cells onto aggregated TiO₂, followed by loss of membrane integrity, is key to the bactericidal effect of photocatalysis.

could be due to the presence of ROS generated by illuminated TiO₂, like O₂^{-•}, H₂O₂ and HO[•], or the illumination (mainly UV) of the cells. Most studies concluded that HO[•] was the main cause of the bactericidal effect of photocatalysis (Ireland *et al.*, 1993; Cho *et al.*, 2005).

The majority of studies conducted to elucidate the mechanism of photo killing of *Escherichia coli* cells with TiO₂ concluded that the membrane attacked first, which causes the cell to die. Sunada *et al.* (2003) proposed that the decomposition of the outer membrane by the photocatalytic reaction leads ultimately to cell death. More specifically, Maness *et al.* (1999) concluded that photocatalysis promoted peroxidation of the phospholipids in the membrane, while Matsunaga *et al.* (1985) and Saito *et al.* (1992) reported that Coenzyme A (CoA) was oxidized resulting in loss of respiratory activity.

HO[•] radicals have an extremely short lifetime (10⁻⁹ s), and must be produced near the membrane if they were to oxidize some of its components. As TiO₂, to our knowledge, is not able to cross the cell membrane, the best fitting scenario would involve binding of the external bacterial cell membrane onto TiO₂ particles, followed by oxidation via the local release of HO[•]. In order to test this idea, we investigated the interaction between bacteria and TiO₂ particles using optical microscopy and flow cytometry.

Materials and methods

Bacterial strain and culture conditions

Escherichia coli K12 wild-type strain MG 1655 (Bachmann, 1987) was grown overnight in Luria–Bertani (LB) broth at 37 °C on a rotary shaker (160 r.p.m.). Aliquots of the preculture were inoculated into fresh medium and incubated in the same conditions to an absorbance at 600 nm of 0.475 ± 0.025 . Cells were harvested by centrifugation at 4330 g for 10 min at 4 °C, washed twice with an NaCl–KCl (8 g L^{-1} NaCl + 0.8 g L^{-1} KCl, $[\text{Cl}^-] = 0.14 \text{ mM}$, $[\text{K}^+] = 0.01$, $[\text{Na}^+] = 0.14 \text{ mM}$, pH 7) solution at 4 °C and resuspended in the photocatalytical solution to a concentration of 2×10^7 cfu mL⁻¹. Culturable bacteria were assayed by plating on LB-agar plates after serial dilution in NaCl–KCl. Colonies were counted after 48 h incubation at 37 °C. Experiments were performed with photoactive TiO₂ (Degussa P25: 20% rutile 80% anatase crystalline form, Degussa AG, Baar, Switzerland) or photoinactive TiO₂ (Huntsman TR 92, 100% rutile crystalline form, Tioxide Europe Ltd, Billingham, UK) at 1 g L^{-1} in the photocatalytical solution. The photocatalytical solution was either composed of NaCl–KCl, pH 7 or sodium phosphate (0.14 mol L^{-1}), pH 5. Indeed, considering that in NaCl–KCl, the pH dropped immediately to pH 5 when the bacteria and TiO₂ were added.

Photocatalytic reaction

Illumination was performed with a xenon lamp in a Hanau Suntest system (AM1) at 550 W m^{-2} light intensity with a filter that cuts off wavelengths below 310 nm. Illumination was also from 310 to 800 nm. The radiant flux was monitored with a Kipp & Zonen (CM3) power meter (Omni-instruments Ltd, Dundee, UK). Experiments were performed in 250 mL Sarstedt polystyrene flasks, closed with septa. Solutions with TiO₂ and bacteria were constantly agitated with a magnetic stirrer at 37 °C. Two syringes were put into the septum: one to allow a direct sampling without stopping the lamp, the other to allow the equilibrium with air. Sampling was performed by taking 2 mL of the reaction mixture with the syringe before and after 5, 10, 20, 40 and 60 min of illumination. For each sampling time, *E. coli* survival was calculated using $[\log(\text{cfu}/\text{cfu}_0); \text{cfu}_0, \text{value at time } 0; \text{cfu value at time } t]$.

Optical microscopy

Microscopy observation was performed with a Zeiss Axiovert 200M microscope (Zeiss, Oberkochen, Germany) with a camera Hamamatsu Orca-ER. All observations were made by phase contrast. 4',6-Diamidino-2-phenylindole (DAPI) staining were performed by mixing cells and TiO₂ with

DAPI (Sigma Chemical Co, Lyon, France) $1 \mu\text{g mL}^{-1}$ in 50% glycerol. Observations were performed by fluorescence (Excitation filter BP 365/12, dichroic mirror FT 395, emission filter LP 397).

Flow cytometry

Samples were analyzed with a flow cytometer (Cytoron Absolute, ORTHO Diagnostic Systems, Issy Les Moulineaux, France) equipped with an air-cooled 488 nm argon laser. The sample and sheath rates were 1 and $100 \mu\text{L s}^{-1}$, respectively. Data were collected and stored in list-mode with the Immunocount software (ORTHO Diagnostic Systems). Cluster analyses were run with WINLIST software (VERITY[®] Software House) (Gregori *et al.*, 2001).

Membrane integrity alteration

Bacteria membrane integrity is a criterion for characterizing viable cells from damaged and membrane compromised cells. Double staining with Sybr Green II (SGII, Molecular Probes[®], Invitrogen, Cergy Pontoise, France) and Propidium Iodide (PI, Sigma Chemical Co) allows the quantification of live cells, membrane compromised cells and dead cells using flow cytometry as described previously by Gregori *et al.* (2001). Samples were also analyzed without staining in order to differentiate the TiO₂ particle background from the adsorbed bacteria cells. Protein detection was performed with the BCA Protein Assay Kit (Pierce, Perbio Science, France, Brebierès, France).

Results

Influence of solution composition on the bactericidal effect of photocatalysis using TiO₂

As illuminated TiO₂ reacts directly or indirectly with many organic compounds found in growth media, we performed inactivation of *Escherichia coli* cells by photocatalysis in sodium phosphate or NaCl–KCl solution (see Materials and methods). Briefly, cells grown to exponential phase were washed, and resuspended in one or the other solution and irradiated in the presence of active or inactive TiO₂. Cell culturability was then evaluated by plating treated cells after serial dilution in NaCl–KCl, and counting the number of colonies formed after 48 h growth. As we found that the pH of the NaCl–KCl solution dropped to pH 5 when cells and TiO₂ were added, we used a sodium phosphate solution at the same final pH. In order to keep irradiation to a minimum and avoid its possible deleterious effects, we favored the use of a high TiO₂ concentration (1 g L^{-1}). As expected, we found that survival of *E. coli* cells decreased upon longer illumination time in either solution (Fig. 1). Interestingly, however, survival was dependent on the nature

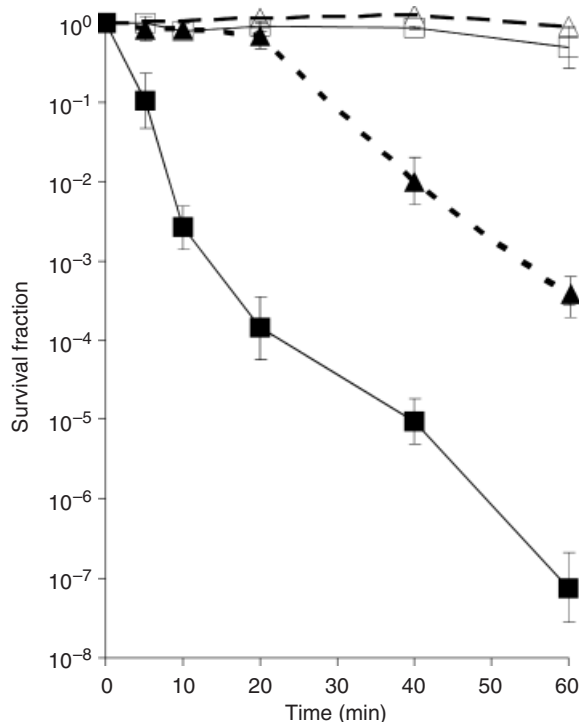


Fig. 1. Influence of solution composition on *Escherichia coli* culturability during illumination (intensity = 550 W m^{-2}) in the presence of TiO_2 . *Escherichia coli* cells ($2 \times 10^7 \text{ cfu mL}^{-1}$) in exponential state were suspended in sodium phosphate solution (\blacktriangle and \triangle) or NaCl–KCl solution (\blacksquare and \square) with TiO_2 (1 g L^{-1}). For each sampling point, $100 \mu\text{L}$ of solution were spread onto Luria–Bertani-agar plate and survival was counted after 48 h. Close symbols represent experiments with photo-active TiO_2 and open symbols with photo-inactive TiO_2 (Hautsman). Points indicate the mean value and standard deviation of five or more independent experiments.

of the solution, as death was evident immediately after illumination in NaCl–KCl, whereas it occurred only after 20 min in sodium phosphate (Fig. 1). This is in agreement with the well established inhibitory property of phosphate upon photocatalysis (Rincon *et al.*, 2004). We note, however, that after 20 min of illumination, the same disinfection kinetics was observed in both solutions, suggesting that photocatalysis is retarded by phosphate and not permanently inhibited. To control the effect of irradiation only, we replaced active TiO_2 with the same amount of inactive TiO_2 . We found that culturability of *E. coli* cells was not affected by inactive TiO_2 after 1 h irradiation in either solution (Fig. 1, open symbols), thus ruling out a major role for irradiation itself in the observed bactericidal effect. Survival was also not affected when cells were incubated in the dark during 24 h in the presence of active TiO_2 and in either solution (data not shown), which indicates that salt composition has no direct influence on cell death. Intriguingly, however, we found that non irradiated cells in contact with 1 g L^{-1} TiO_2 in sodium phosphate or NaCl–KCl solutions are three times

less culturable if subsequently diluted in sodium phosphate instead of NaCl–KCl before plating (data not shown). As this was not the case in absence of TiO_2 , we suggest a possible interaction between salt, bacteria and TiO_2 .

Overall, our results suggest that *E. coli* death is due primarily to TiO_2 -based photocatalysis, which is more readily obtained in NaCl–KCl solution.

Interaction between bacteria and TiO_2 particles without illumination ($t = 0 \text{ min}$)

Our data pointed at possible differential interactions between *E. coli* cells and TiO_2 depending on the solution used. We directly addressed this issue by analyzing with optical microscopy the adsorption of bacteria on the catalyst without illumination ($t = 0 \text{ min}$) in either of our buffers. We did not observe free bacteria in NaCl–KCl solution (Fig. 2a), as indeed all of them appeared to be adsorbed onto TiO_2 particles (Fig. 2b). We noted that single titanium dioxide particles, which had a variable size (data not shown), could adsorb several cells (Fig. 2b). In contrast, little adsorption onto TiO_2 particles was seen in sodium phosphate solution, and most bacteria were free-swimming (Fig. 2c). We independently confirmed these results by flow cytometry using SG II fluorescence (see Materials and methods). As shown in Fig. 3, without illumination, most bacteria were adsorbed on TiO_2 in NaCl–KCl, but not in sodium phosphate solution. These observations provide a possible explanation to the delayed inactivation of *E. coli* by photocatalysis in

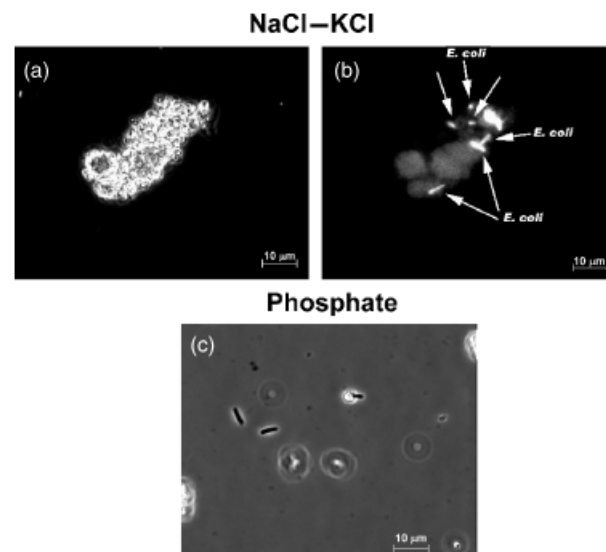


Fig. 2. Optical microscopy of *Escherichia coli* cells in presence of TiO_2 in different solutions. For the sample in NaCl–KCl, bacteria, stained with 4',6-diamidino-2-phenylindole, were visualized by (a) phase-contrast and (b) fluorescence microscopy. In sodium phosphate solution (c), the bacteria are not stained and image is visualized by phase-contrast.

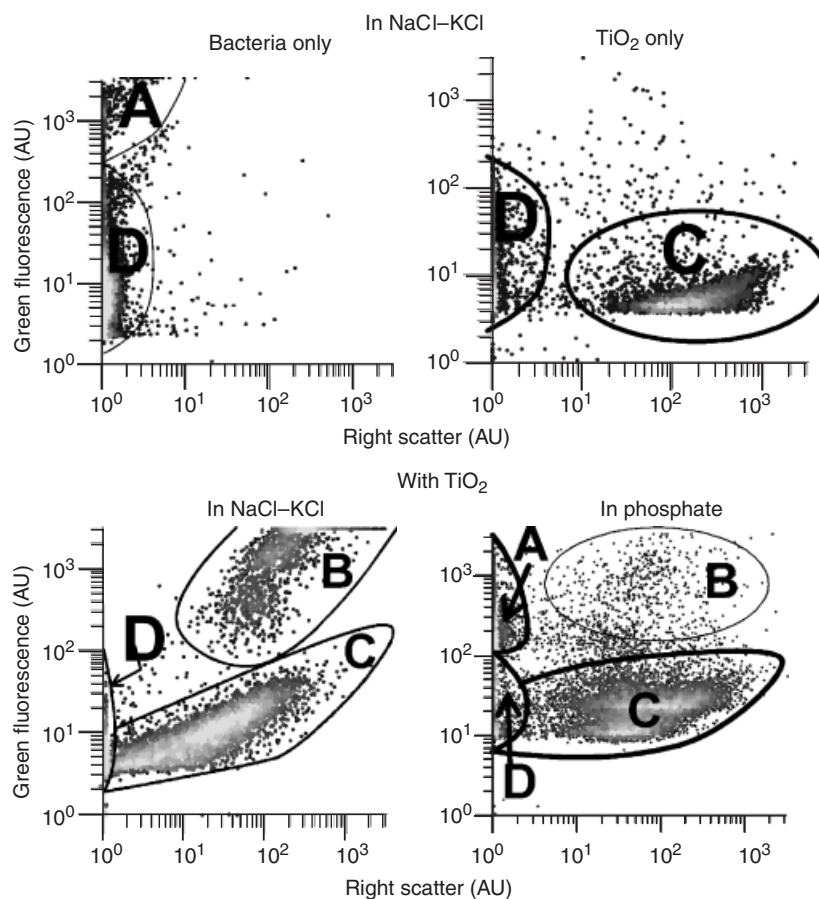


Fig. 3. Interaction between *Escherichia coli* and TiO₂ aggregates without illumination ($t = 0$ min): influence of solution composition. Zone A corresponds to free bacteria, zone B to bacteria adsorbed on TiO₂, zone C TiO₂ aggregates without bacteria and zone D to noise.

sodium phosphate, as this salt is apparently not favorable for the interaction between TiO₂ and the cell.

Adsorption is involved in the bactericidal effect

If the link between adsorption and loss of culturability hypothesis is correct, we expect adsorption to be delayed when photocatalysis is performed in sodium phosphate, as was the case for inactivation (Fig. 1). We thus studied the kinetics of adsorption of bacteria onto TiO₂ particles over 60 min of illumination in sodium phosphate solution. Using flow cytometry, we were able to calculate the percentage of adsorbed cells using the number of free bacteria (Fig. 4, zone A) and the number of bacteria bound to TiO₂ (Fig. 4, zone B). Considering that microscopic observations indicated that TiO₂ particle sizes were below the bacterial size and that one TiO₂ particle adheres to no more than one bacterial cell, therefore, for each TiO₂ particle there is a bacterial cell. Taking this observation into account, we assume that events observed in zone B is equal to adsorbed bacteria. Moreover, when the sum of events from A and B were calculated, it equalled the initial bacterial concentration (2×10^7 cells per mL), indicating indeed that each event detected in zone B is due to only one bacteria.

We found indeed that adsorption of bacteria was weak below 20 min of illumination, whereas after 60 min more than 75% were adsorbed (Fig. 4). These results were confirmed by optical microscopy (data not shown). Interestingly, we noticed that TiO₂ aggregates are small in sodium phosphate solution compared with those in NaCl-KCl (data not shown), and that small aggregates seem to adhere to bacteria (Fig. 3).

Consistent with the above hypothesis, we could confirm that culturability started to decrease when cells became adsorbed on the catalyst, strongly suggesting that adsorption onto TiO₂ is a prerequisite for bacterial inactivation (Fig. 4).

First step of the bactericidal effect: alteration of membrane integrity

Having established that adsorption is tightly linked to initiation of the bactericidal effect, the next obvious step in our study consisted in analyzing membrane integrity during illumination (Sunada *et al.*, 2003). For this purpose, we used flow cytometry with a double DNA staining procedure allowing us to measure cell viability as a function of cytoplasmic membrane integrity (Barbesti *et al.*, 2000). We observed in Fig. 5 that, in NaCl-KCl solution, mortality and

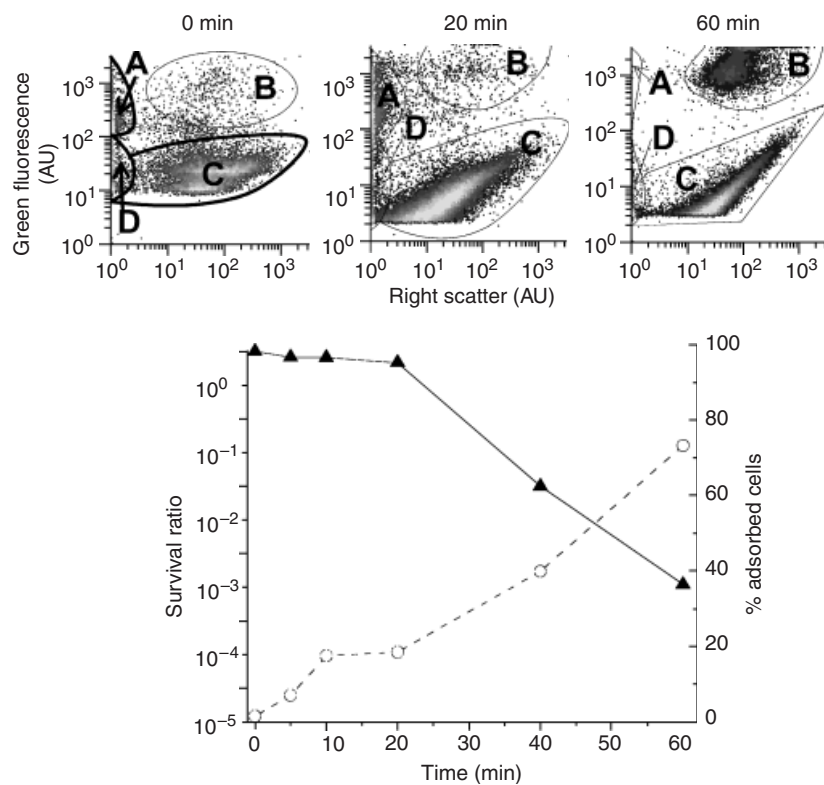


Fig. 4. Evolution of *Escherichia coli* adsorption onto TiO₂ during illumination in sodium phosphate solution. Green fluorescence (arbitrary units) vs. right scatters (arbitrary units) cytogram: zone A represents free bacteria, zone B bacteria adsorbed on TiO₂ aggregates, zone C TiO₂ aggregates without bacteria and zone D noise. *Escherichia coli* (2×10^7 cfu mL⁻¹) is in sodium phosphate solution (pH 5, 0.15 mol L⁻¹) in presence of 1 g L⁻¹ TiO₂. The graph shows the *E. coli* survival ratio (▲) and percentage of adsorbed cells (○) vs. illumination time.

by inference the loss of membrane integrity was drastic: after 4 min, most cells could be considered as nonviable. In contrast, when staining was performed on cells illuminated 20 min in sodium phosphate solution, a large proportion was still alive. Moreover, the number of viable cells corresponded well with the number of culturable cells. After 40 min of illumination, however, most bacteria were nonviable suggesting that major membrane damages had occurred. Interestingly, we found that 60% and less than 5% of the cells in NaCl–KCl and sodium phosphate solutions respectively, displayed altered membrane integrity before illumination. In the case of NaCl–KCl, protein detection in supernatant of cells exposed to TiO₂ indicated that cytoplasmic membranes started to disintegrate even before illumination (data not shown). This suggests that adsorption alone without illumination already alters membrane integrity, a phenomenon greatly amplified when illumination occurred.

Discussion

The bactericidal effect of photocatalysis with TiO₂ is well established but its mode of action is unclear. It is believed that HO produced by TiO₂ upon illumination constitutes the primary killing agent (Cho *et al.*, 2005). Because of the short half life of HO[•] and its low diffusion potential, bacterial targets to be oxidized must be close to the place

where HO[•] is generated, which in our case is TiO₂ particles. This hypothesis implies adsorption of bacteria onto TiO₂, which surprisingly has never been reported in the literature. Here, we show for the first time that adsorption of bacteria on TiO₂ aggregates is essential for the bactericidal effect of photocatalysis.

Our work also indicates that bacterial adsorption is influenced by two parameters, composition of the solution used during photocatalysis, and duration of illumination. Indeed, while all cells were bound to TiO₂ aggregates in sodium phosphate solution, less than 5% of cells adhered to TiO₂ aggregates in NaCl–KCl solution, whereas death was much delayed in sodium phosphate solution, we argue that aggregation of bacteria onto TiO₂ particles is a driving force for the bactericidal effect. Microscopic observations suggested that, before illumination, only small TiO₂ aggregates were present in sodium phosphate solution, whereas large aggregates formed in NaCl–KCl solution that did or did not contain bacteria. This suggests that phosphate ions prevent TiO₂ aggregation leading to the formation of only small aggregates. In agreement with this idea, Saito *et al.* (1992) reported that TiO₂ particles are charged positively at pH 5 and can bind negative ions like Cl⁻ and HPO₄²⁻ or bacteria. However HPO₄²⁻ is much more affine than Cl⁻ against TiO₂ (Chen *et al.*, 1997) which may explain why bacteria were

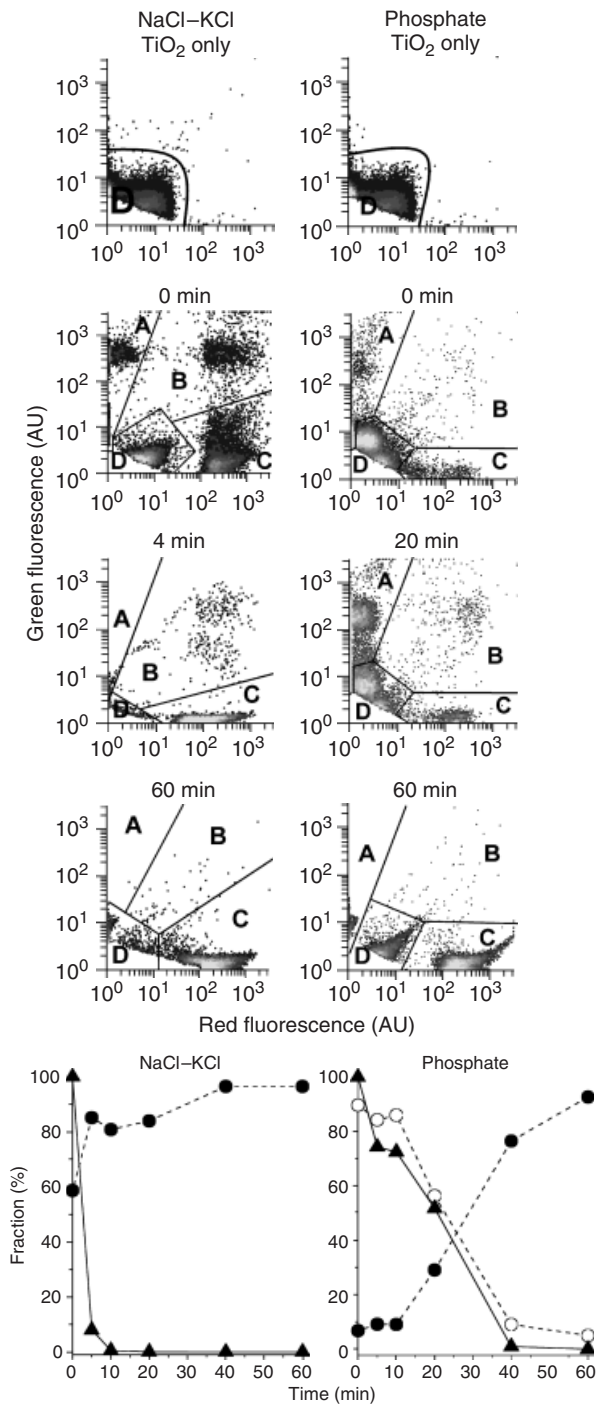


Fig. 5. Evolution of *Escherichia coli* viability during illumination in the presence of TiO₂. Bacteria were double stained with SYBR Green I (green fluorescence) and with propidium iodide (red fluorescence) fluorochrome. Signals are distributed in four zones: zone A represent viable cells, zone B cell with partially damaged membrane, zone C nonviable cells and zone D noise. The graph shows the *E. coli* survival ratio (\blacktriangle), the percentage of viable cells (zone A) (\circ) and the percentage of nonviable cells (zone C) (\bullet), vs. illumination time.

more adsorbed in NaCl–KCl solution than in sodium phosphate one. During the course of illumination, we could clearly observe that the size of TiO₂ particles increased also in sodium phosphate solution (data not shown). This phenomenon likely implicates phosphate desorption, allowing in turn the TiO₂ particles to recover their aggregation capacity. This may explain why, in sodium phosphate solution, bacterial adsorption on TiO₂ aggregates increases upon prolonged illumination. As TiO₂ is a semi-conductive catalyst, its physico-chemical properties may vary greatly when it is illuminated, which can lead to photoadsorption and/or photodesorption (Linsebigler *et al.*, 1995). For instance, Yoo *et al.* (2004) showed that upon application of UV light, AgNO₃ molecules were totally adsorbed on TiO₂ within 120 min, which was not the case in the dark. They also noted that adsorbed AgNO₃ showed a more significant photocatalytic reaction than free AgNO₃, which again illustrates the importance of adsorption. Also relevant from our study, Bredemeyer *et al.* (2000) showed that irradiation intensity and temperature decreases the adsorption isotherm of NO on TiO₂. On the basis of these chemical studies, we would like to propose the following scenario for TiO₂-based photocatalysis in sodium phosphate solution. Initially, TiO₂ binds with high affinity to phosphate and exhibits little aggregation. Upon prolonged illumination, increasing photodesorption of phosphate occurs as its affinity for TiO₂ decreases, allowing bacteria to adhere.

We report that the bacterial adsorption rate on TiO₂ upon illumination is tightly linked to loss of *E. coli* viability, in either solution used in this study. In keeping with the importance of adsorption, it is known that HO[•] radicals formed on the TiO₂ surface display a higher bactericidal efficiency than those free in the solution (Cho *et al.*, 2005). However, we do not rule out a more direct effect of TiO₂ as an oxidant. Indeed, with its positive ‘holes’ (h_{vb}⁺), TiO₂ may have on bacteria a direct bactericidal effect, which would also necessitate the contact with the cells. The bacterial adsorption associated with the low diffusion rate of HO[•] radicals or positive ‘holes’ (h_{vb}⁺) point at the cytoplasmic membrane as the primary target in the killing process, as often suggested in the literature. In this study, we observed that viable cells, measured by membrane integrity, were close to the culturable count, suggesting that no viable-but-nonculturable cells were generated during photocatalytical treatment with TiO₂. However, we noted that a number of cells with compromised membranes in NaCl–KCl solution without illumination (recorded as nonviable cells), were actually viable as evidenced by culturability. This phenomenon could not be observed when sodium phosphate solution was used. We conclude that interaction between TiO₂ and adsorbed bacteria modifies membrane permeability in the absence of illumination, without directly causing cell death.

The production of extremely reactive hydroxyl radical by TiO₂ photocatalysis combined to membrane alteration via adsorption, explains the high efficiency of this disinfection procedure. We believe that our work yields important insights to further improve and implement this technique in the future.

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