Published in final edited form as:

Environ Sci Technol. 2010 September 15; 44(18): 6992–6997. doi:10.1021/es101343c.

Visible-Light-Induced Bactericidal Activity of Titanium Dioxide Co-doped with Nitrogen and Silver

Pinggui Wu^{1,#}, Rongcai Xie^{1,@}, Kari Imlay², and Jian-Ku Shang^{1,*}

¹Department of Materials Science and Engineering, University of Illinois at Urbana-Champaign, Urbana, IL 61801 (USA)

²Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, IL 61801 (USA)

Abstract

Titanium dioxide nanoparticles co-doped with nitrogen and silver (Ag₂O/TiON) were synthesized by the sol-gel process and found to be an effective visible light driven photocatalyst. The catalyst showed strong bactericidal activity against *Escherichia coli* (*E. coli*) under visible light irradiation ($\lambda > 400$ nm). In x-ray photoelectron spectroscopy and x-ray diffraction characterization of the samples, the as-added Ag species mainly exist as Ag₂O. Spin trapping EPR study showed Ag addition greatly enhanced the production of hydroxyl radicals ($\bullet$ OH) under visible light irradiation. The results indicate that the Ag₂O species trapped e_{CB}^- in the process of Ag₂O/TiON photocatalytic reaction, thus inhibiting the recombination of e_{CB}^- and $h\nu_B^+$ in agreement with the stronger photocatalytic bactericidal activity of Ag₂O/TiON. The killing mechanism of Ag₂O/TiON under visible light irradiation is shown to be related to oxidative damages in the forms of cell wall thinning and cell disconfiguration.

Introduction

Adequate, reliable, and environmentally safe disinfection is of great significance since regulatory agencies have established and enforced more and more rigid bacteriological effluent standards. In seeking an alternative technique to avoid disinfection by-products of chlorination, in 1985, Matsunaga et al. (1) discovered the bactericidal activity of TiO₂ as a photocatalyst. Since then, the bactericidal activity of TiO₂ has been of significant importance for many applications across several fields, from purification of air (2) and water (3–5) to the sterilization of food (6) and hospital utensils (7). Various organisms have been photocatalytically inactivated by TiO₂, including bacteria (6–10), bacterial and fungal spores (11–13), and algae (14).

Traditional TiO₂ photocatalysis is effective only upon irradiation of UV-light at levels that would induce serious damage to human cells (15). To overcome this limitation, researchers have conducted extensive study on doping and sensitization effects in TiO₂. Many individual doping elements, such as nitrogen (16–18), sulfur (19), carbon (20), etc., are found to induce visible light photo-activity. Yet the visible-light-induced photocatalytic efficiency of the modified TiO₂ is often found not high enough. For example, Yu and co-

*Corresponding author, jkshang@illinois.edu.

#Currently with Superior Graphite Co., Chicago, IL 60632. pwu@superiorgraphite.com

@Currently with Institute of Metal Research, Chinese Academy of Sciences, Shenyang, 110016, P. R. China.

Supporting Information Available

XPS spectra, more bactericidal data, EPR spectra of TiO₂ and the Fenton Reaction product, more SEM and TEM images of treated *E. coli* are available free of charge via the Internet at <http://pubs.acs.org>

workers (19) published a report on the visible-light-induced bactericidal effect of sulfur-doped nanocrystalline TiO₂. The survival ratio of *Micrococcus lylae* (gram-positive) decreased to *ca.* 64% after 30 min, and to *ca.* 3% after 60 min radiation.

To improve the low photocatalytic efficiency of single-element doped TiO₂, doping with two or more elements received more attention recently (21–24). For example, the combination of Pd ion and nitrogen resulted in a visible-light activated PdO/TiON photocatalyst, which has shown remarkable photocatalytic activities on a wide range of organic (25) and microbiological species, including virus (26), bacteria (8–10) and spore (11). The addition of PdO allows the electron transfer process (27) on the photocatalyst to be “regulated” by storing and releasing electrons to minimize electron-hole recombination or to produce a long-lasting photocatalytic ‘memory’ effect after light is turned off (28–29). However, PdO/TiON photocatalyst has no bactericidal activity in the initial no-irradiation condition (9), and the bactericidal activity in the dark from the “memory” effect is much weaker than that in the irradiated state (28).

Since Ag ion is a known bactericidal agent (30) and may go through the similar change in the valence state as Pd ion does, Ag ion was used to modify nitrogen doped TiO₂ (TiON) to explore its potential in controlling electron-hole recombination on TiON photocatalyst, and consequently in enhancing the bactericidal activity of TiON. Indeed, Ag₂O/TiON was found to generate significantly greater amount of hydroxyl radicals and exhibit a much stronger photocatalytic bactericidal effect than TiON against *E. coli* under visible light irradiation. Different from PdO/TiON, Ag₂O/TiON also shows anti-bacteria effect in the dark due to the presence of Ag species. This attribute is obviously desirable when sunlight is weak or not available at times.

Experimental Section

Chemicals and Materials

Chemicals were purchased from Sigma-Aldrich, St. Louis, MO unless stated else. Titanium tetraisopropoxide (TTIP, 97%), tetramethylammonium hydroxide (TMA, 25 wt % in methanol), and silver acetylacetonate (Ag(acac), 98%) were used in this study as sources of titanium, nitrogen, and silver, respectively. Ethyl alcohol (EtOH, 100%, AAPER Alcohol and Chemical Co., Shelbyville, KY) and dichloromethane (CH₂Cl₂, 99.6%) were used as solvents.

Sol-Gel Process

Ag₂O/TiON photocatalysts were prepared at room temperature by the following sol-gel process. First, TMA was dissolved in EtOH at a mol ratio at 1:50. The solution was stirred magnetically for 5 min, and then TTIP was added into the solution at a TMA:TTIP molar ratio of 1:10. A proper amount of Ag(acac) was dissolved in CH₂Cl₂, and then added into the TMA/TTIP/EtOH mixture to achieve a target Ag:Ti molar ratio at 0.5%. The mixture was loosely covered and stirring continued until a homogeneous gel formed. The hydrolysis of precursors was initiated by exposure to the moisture in air. The gel was aged in air for 24 h to allow further hydrolysis and drying. Then after drying in 60°C oven, the xerogel was crushed into fine powders and calcined at 400°C in air for 3 h to obtain the desired fine crystallites Ag₂O/TiON. TiON was prepared in a similar manner without Ag(acac).

Characterization of photocatalysts

In x-ray diffraction (XRD), a Rigaku RAX-10 x-ray diffractometer was run at Cu K α radiation (45kV, 20mA). X-ray photoelectron spectroscopy (XPS) measurements were performed on a Physical Electronics PHI 5400 X-ray Photoelectron Spectrometer (Perkin-

Elmer) with a Mg K α anode (15 kV, 400W) at a take-off angle of 45°. The UV-Vis optical spectra of representative photocatalysts were recorded on an HP8452A diode array spectrometer with a deuterium source in the range of 190 nm – 820 nm at 2 nm readout per diode. A reflectance assembly was custom-built using two optical mirrors for the measurements of solid samples. The detection of hydroxyl radicals by spin trapping electron paramagnetic resonance (EPR) followed the addition of 0.2 ml 100 mM α -(4-pyridyl-1-oxide)-N-tert-butyl nitron (POBN) and 0.02 ml 95% ethanol into the irradiated photocatalyst suspension without bacteria. EPR spectra were collected on a Eline Century Series EPR Spectrometer (Varian E-109-12) working in the X-band mode at 9.51 GHz, center field 3390 G, and power 20 mW.

Photocatalytic inactivation of bacteria under visible light

The cultivation and processing of *E. coli* AN 387 cells and experimental set-up for a static bactericidal test are the same as previously published for PdO/TiON (9): aliquot of 3-ml *E. coli* cell suspension was pipetted onto a sterile petri dish with photocatalyst dispersed in the suspension. The light source is a metal halogen desk lamp with a filter (> 400 nm), light intensity ~ 1.6 mW/cm². At regular time intervals, 20 μ L aliquots of the irradiated cell suspensions were withdrawn. After appropriate dilution (26 $\times$ 26, or 26 $\times$, or 1 $\times$) in buffer, aliquots of 20 μ L together with 2.5 ml top agar were spread onto agar medium plates and incubated at 37°C for 18–24 h. The number of viable cells in terms of colony-forming units was counted. Analyses were duplicated and control runs were carried out under the same irradiation condition to cell suspension without a photocatalyst. Comparison in the non-irradiation condition of Ag₂O/TiON was also performed.

Scanning electron microscopy (SEM)

Overnight grown *E. coli* cells prior to photocatalytic treatment and after treatment were collected by centrifugation. The pellet was fixed in 2.5% glutaraldehyde for 2 h in a refrigerator. After fixation, the cell pellets were soaked in cacodylate buffer to remove excess fixative. Post-fixation processing was carried out in 1% osmium tetroxide in cacodylate buffer for 90 min at room temperature, and the pellets were then washed with cacodylate buffer. The samples were dehydrated by successive soakings in 37, 67, 95% (v/v) ethanol for 10 min each and then three soakings in 100% ethanol for 15 min each. Critical point drying was performed by placing samples in hexamethyldisilazane (HMDS) for 45 min and overnight drying under a fume hood after drawing the HMDS off. SEM images of the samples were obtained using a scanning electron microscope Hitachi S-4700 (Hitachi, Tokyo, Japan) at an acceleration voltage 5 or 10 kV.

Transmission Electron Microscopy (TEM)

Overnight-grown *E. coli* cells were centrifuged prior to or after photocatalytic treatment. The collected *E. coli* cell pellet was processed and TEM images were taken by specialists in the Center for Microscopic Imaging (CMI) of the College of Veterinary Medicine, University of Illinois at Urbana-Champaign. The pellet was fixed in Kamovsky's fixative at refrigerator temperatures for a minimum of 3 h until processing. Microwave techniques were used for fixation and other steps in the procedure. The sample was first washed with cacodylate buffer and secondarily fixed in 2% osmium tetroxide, followed by the addition of potassium ferrocyanide. The sample was then washed in water and enbloc stained with uranyl acetate. The cells were dehydrated by successive incubations in 25, 50, 75, and 95% (v/v) ethanol for 8 min each, two incubations in 100% ethanol, and finally two incubations in 100% acetonitrile. The sample pellets were then infiltrated with a mixture of epoxy resin and acetonitrile (1:1 v/v) for 10 min, a mixture of epoxy resin and acetonitrile (4:1 v/v) for 20 min, and finally pure epoxy resin for 3 h at room temperature. Following infiltration, the sample was placed in individual embedding capsules, spun down to a pellet, and then

polymerized at 85°C overnight. These samples were removed from the capsules and trimmed. Ultrathin sections (60–90 nm) were mounted on copper grids and stained with uranyl acetate and lead citrate. TEM images were taken with a Hitachi H600 transmission electron microscope operated at 75 kV.

Results and Discussion

Crystal structure of the photocatalysts

The obtained solgel powders of Ag₂O/TiON are shallow gray, finely crystallized. Figure 1 demonstrates the XRD patterns of Ag₂O/TiON and TiON particles, respectively. Both show that the main XRD peaks belong to the typical anatase phase with no rutile phase observed. Apparently, incorporation of a small amount of the dopant from the solgel process does not alter the crystal structure of the TiO₂ powders. A weak XRD peak assigned to Ag₂O (101) (31–32) could be identified in the XRD pattern of Ag₂O/TiON powder sample. This observation suggests that the silver additive exists as Ag₂O in the Ag₂O/TiON catalyst with rather small quantity of Ag₂O. It appears that the silver additive is not incorporated into the main anatase crystalline structure.

Composition of the photocatalysts

Semiquantitative analyses on the chemical ingredients of Ag₂O/TiON photocatalyst was performed in XPS. Figure S1 (see Supporting Information) demonstrates the presence of N, O, Ag, and Ti in the powder sample. Multiplex scans were performed for the peaks of N1s, Ag3d, O1s, and Ti2p respectively. The N1s peak has a binding energy of ~399.5 eV, which indicates that the nitrogen in solgel-obtained Ag₂O/TiON is not in the atomic state, and suggests that some O atoms in the TiO₂ structure are substituted by N atoms to form Ti-N bonding. The binding energy of Ag3d_{5/2} is ~367.9 eV and Ag3d_{3/2} ~373.4 eV, which can be attributed to Ag₂O species (31), in agreement with the XRD result. Semiquantitative composition data were obtained from analyzing these high-resolution scans, using the built-in software to compare relative peak intensities and atomic sensitivity factors. The data indicate low incorporation of nitrogen and silver in the Ag₂O/TiON catalysts: silver content is ≤ 0.5 at.%, and nitrogen ≤ 2 at.%. These estimates considered the XPS data of this experiment as well as the data of other Ag₂O/TiON catalysts prepared at higher precursor concentrations (31).

Optical properties

Figure 2 shows the light absorbance of Ag₂O/TiON particles, compared with the light absorbance of TiON. TiON powders were prepared through the same process as Ag₂O/TiON samples except for the addition of Ag(acac). A commercial TiO₂ sample Degussa P25 powder was used as the reference material in this study. Degussa P25 has an absorption stopping edge at 395 nm (31), which is in accordance with the observation that its photocatalytic activity is restricted mainly to the ultraviolet light region. TiON powders show a clear shift into the visible light range (>400 nm) owing to the nitrogen doping effect (18). Ag₂O/TiON absorbance plot shows a higher visible-light shift than that of TiON powder, to 450 nm and beyond. The comparative data suggest that silver additive promotes visible light absorption in the nitrogen-doped TiO₂ sample. The co-doped Ag₂O/TiON powder has a great deal of optical absorbance in the visible light region.

Disinfection kinetics

Figure 3 shows the gradual deduction of colony counts in auger medium plates after treatment. The sterilization tests indicate that the irradiation (control sample, irradiated without photocatalysts) has no bactericidal effect. On the contrary, the bactericidal function

of Ag₂O/TiON started in the first time interval, and became more and more evident with longer irradiation time. Since silver and silver ion have long been known to have antibacterial activity (30), comparison tests using irradiated commercial silver powder (Sigma-Aldrich, 99.5% trace metal basis) and Ag₂O/TiON powder in the dark were also conducted, all at concentration 1.3 mg/ml. Neither of the two tests showed a comparable killing rate as the irradiated Ag₂O/TiON. Ag₂O/TiON powder under visible light irradiation shows faster sterilization toward *E. coli* than Ag₂O/TiON in the dark and the irradiated Ag powder. Since the silver ion content is extremely low in the Ag₂O/TiON powder, it can be deduced that the anti-bacteria activity of irradiated Ag₂O/TiON is mainly attributed to photocatalytic reaction. Previously, some photocatalysts based on silver-titania were reported by other groups (33–34). Results of those studies indicate that a silver-doped TiO₂ material is not photocatalytically active under visible light irradiation, especially when the loaded silver is at low concentrations. In Figure S2 of Supporting Information, a series of bactericidal tests found that concentrated Ag₂O/TiON in the bacteria suspension may not necessarily be the optimal application condition. Ag₂O/TiON powder aggregation or less efficient absorption of irradiation can be associated with more Ag₂O/TiON powder. It also suggests that the photocatalytic anti-bacteria effect is more important than the contribution of the antibacterial effect from the silver ions in Ag₂O/TiON.

Because there are no standardized conditions for photocatalytic inactivation of *E. coli* cells, a direct comparison between the present data with previously reported photo-sterilization activity of TiO₂ under UV or that of doped TiO₂ under visible light irradiation is not realistic. However, the survival fraction $<10^{-5}$ within *ca.* 30 min is one of the fastest disinfection rates of *E. coli* inactivation ever reported using TiO₂-based photocatalyst (1,9,19). Compared to our previously reported PdO/TiON photocatalysts, the antibacterial activity of irradiated Ag₂O/TiON powder seems to outperform that of PdO/TiON powder under similar conditions (9); yet, it was lower than that of PdO/TiON nanoparticles well-dispersed on a fiber (9) or a monolithic PdO/TiON ceramic foam (8). It suggests that the enhancement effect of these transition metal ions is dependent on the type of the metal ion, as well as the physical form of the photocatalyst (31).

Hydroxyl radical production on irradiated Ag₂O/TiON photocatalyst

To verify that hydroxyl radicals may be produced in the photocatalytic process and thus responsible for inactivation of microorganisms, spin trapping EPR measurements were conducted on the Ag₂O/TiON particle powder. To investigate the silver ion modification effect on the production of reactive oxygen species in the photocatalytic process, the spin trapping EPR spectrum of TiON particle powder was also measured. Figure 4 shows the spin trapping EPR spectra of various photocatalyst samples in interaction with POBN under visible light irradiation for different time periods. The peaks in EPR spectra are characteristic of the POBN-OH• spin adduct (29,31). It can be seen that at the start point of visible-light-activated photocatalytic reaction on Ag₂O/TiON powder, spin trapping EPR signal was barely detected. After Ag₂O/TiON powder was irradiated for 5 minutes, the characteristic peaks of the POBN-OH• spin adduct were observed. The peak intensities of 10-min and 30-min irradiated Ag₂O/TiON photocatalytic system obviously increased with the irradiation time, indicating the production of OH• has an accumulation effect. These results confirm the production of hydroxyl radicals in the Ag₂O/TiON photocatalytic process under visible light illumination. The spin trapping EPR characteristics of POBN-OH• are also observed on visible-light irradiated TiON powder; however, the peaks are rather weak. This observation suggests that TiON has photocatalytic activity upon visible-light irradiation due to its visible light absorption capability (Figure 2). The low photocatalytic efficiency of TiON could be caused by the electron-hole charge carrier recombination problem. On the basis of the enhancement effect of silver ion modification, it

is believed that the silver ion has served as electron trappers and effectively reduced the hole-electron recombination rate, largely increasing the production of hydroxyl radicals. The role of silver in the TiON is thus similar to those reports of silver in Ag-loaded TiO₂ (35) and Ag/AgBr/TiO₂ photocatalysts (34). Prior to obtaining the reported spectra, a Fenton reaction was conducted to verify the methodology and the spectrum of Degussa P25 TiO₂ was measured to be a control. Results are shown in Figure S3 (see Supporting Information). The Fenton showed high-intensity POBN-OH• signal, while no POBN-OH• signal was observed under visible light irradiation of TiO₂.

Evidence of oxidative damages

In Figure 5(a) a representative SEM image of *E. coli* cells before photosterilization treatment was illustrated. In this control sample, the surfaces of rodlike bacteria are smooth and damage-free. It indicates that the cells were healthy before they were treated with Ag₂O/TiON photocatalyst. However, after complete inactivation of the bacteria cells under visible-light illumination of Ag₂O/TiON for 2 h, the morphology of these cells showed dramatic changes. First, the flagella observed in untreated cells were completely missing in Figure 5(b) and Figure S4 (supporting information) of treated cells. Second, in nearly every cell the appearance of rumples and a high degree of disconfigurations were observed. Images in Figure 5(b) and S4 show that many *E. coli* cells were subject to mass-missing on the cell wall and the cell membrane or even material inside, so that deep 'holes' appeared. These images verified that photocatalysis caused oxidative damages on bacteria. The formation of rumples/holes in *E. coli* was in good agreement with some previous reports (36).

Most of the existing discussions of photocatalytic killing mechanism are based on UV/TiO₂ systems. Different mechanisms of killing have been proposed, including these three major proposals: a) detrimental effects on deoxyribonucleic acid (DNA) molecules (37); b) cell wall and cell membrane damage (38) that leads to leakage of the cell contents (39); c) observed decrease or loss of respiratory activities due to oxidation/loss of coenzyme A (1). TEM images concurred with the previous SEM observation of membrane damage. Figure 6(a) is a representative TEM image of untreated *E. coli* cells that have a fluffy boundary. The fluffy outer layer is considered to be the outer membrane of *E. coli* cell. It can be noted that after photocatalytic inactivation, the outer membrane had completely decomposed. In Figures 6(b) and Figure S5 (Supporting Information), a noteworthy difference from the untreated sample is that every treated cell has lost its outer membrane, i.e., the fluffy edge. Some treated cells show a clearly-cut edge, which indicates that the plasma membrane may have been exposed after the outer membrane is decomposed. The other cells completely or partially lost this edge, which is a severely damaged stage with plasma membrane also gone. The damage to the cell wall and the cell membrane observed in TEM are in good agreement with the SEM results.

TEM images also show remarkable interior damage on cells after photocatalytic disinfection. Normal *E. coli* cells exhibit a homogeneous microstructure in Figure 6(a). Characteristics of the *E. coli* cell are a well-defined cell wall as well as the evenly colored interior, which corresponds to full of proteins and DNA molecules (30, 34). We can see that the healthy cells show uniform interior material density in the TEM image 6(a). In contrast, dark mass aggregates appeared in the treated cells in Figures 6(b) and S5. In some more severely compromised cells, such as two cells in Figures 6(b), white center regions were observed.

The appearance of these white-color areas may result from several possible events. One could interpret the white areas to be aggregated DNA molecules (30). It is fair to conjecture that the gross morphology of the DNA may have changed, meaning that its higher-order organization may have been disrupted upon the impact of photocatalysis, such as reported

elsewhere (30,37). Another interpretation of the phenomenon is the leaking of interior components after rupture of the cell membrane (34). Another alternative explanation might be the decomposition of interior components upon oxidation by reactive oxygen species. Although evidence should be sought to provide a solid answer, the first interpretation is more likely to be the case than the others. Reactive oxygen species generated in the present experimental set-up is mainly hydroxyl radicals. They are known to be too active to survive a long-range diffuse through the compromised cell wall/cell membrane and reach the cell center. Furthermore, if leaking or decomposition of the interior components occurs, it is more likely to start with the border areas near the cell membrane, not the center. The areas surrounding the cell membrane shall suffer the most severe consequences, not the center. On these bases, we interpret the mechanism of visible light photocatalytic bactericidal activity of Ag₂O/TiO₂ to be oxidative damages to the cell wall and the cell membrane of *E. coli*, followed by immediate serious impact on the interior DNA molecules.

In summary, we demonstrated that Ag ion modification of nitrogen-doped TiO₂ photocatalyst preserved the option of using visible light to activate TiO₂ but greatly enhanced its photocatalytic activity. The primary role of Ag ions in enhancing the bactericidal activity of Ag₂O/TiO₂ under visible light illumination was photocatalytic in promoting the production of hydroxyl radicals rather than acting as the bactericidal agent by themselves. The prevalent mechanisms for the photocatalytic killing of *E. coli* consisted of the oxidative damages on the cell wall and the cell membrane, and alterations of the internal DNA molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Dr. Mark Niegl for the EPR discussion and characterization, thank Lou Ann Miller for the TEM characterization work. This material is based upon work supported by the Center of Advanced Materials for the Purification of Water with Systems, National Science Foundation, under Agreement Number CTS-0120978. XPS and XRD were carried out at the Frederick Seitz Materials Research Laboratory, University of Illinois at Urbana-Champaign, which is partially supported by the U.S. Department of Energy under grant DEFG02-91-ER45439.

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Figure 1. Powder XRD patterns of TiON and Ag/TiON, respectively (A, anatase). The possible Ag-oxide peak is marked with an asterisk (*).

Figure 2. UV-Vis absorption spectra of Ag₂O/TiON (top) and TiON (bottom) powder. The dash line represents the typical absorbance stopping edge of a Degussa P25 TiO₂ powder.

Figure 3. Survival ratio of *E. coli* versus irradiation time in the suspension of TiON (open square), commercial Ag powder (▼), Ag₂O/TiON at dark (■) or irradiated (▲); the solid circle are control data. (Note: the lines merely guide the eyes.)

Figure 4.

Spin trapping EPR spectra of the spin adduct POBN-•OH resulted from the Ag₂O/TiON system for visible-light irradiation time 0, 5, 10, and 30 min, compared to visible-light irradiated TiON for 10 min. In TiON, the adduct peaks are marked to guide the eyes.

(a)

Figure 5. SEM images of (a) untreated *E. coli* cells; (b) well-treated *E. coli* cells upon visible light illumination in the presence of Ag^+/TiON powder for 2h.

(a)

(b)

Figure 6. TEM images of (a) untreated; (b) well-treated *E. coli* cells. Treatment is visible-light illumination upon Ag^+/TiON powder for 2h—dark granules are observed and cells have damaged cell membrane.