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Disruption of Bacterial Cells by Photocatalysis of Montmorillonite Supported Titanium Dioxide

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Abstract: The photo-induced antibacterial capacity of montmorillonite supported titanium dioxide (TiO_2/Mmt for short) was evaluated by using *Escherichia coli* and *Staphylococcus aureus* as modal organisms. The bactericidal activity of TiO_2/Mmt was examined by cell viability assay under different illumination modes. Atomic force microscopy (AFM) and total organic carbon/Total nitrogen (TOC/TN) analyses were employed to investigate the mechanism of the photocatalytic bactericidal process qualitatively and quantitatively. The kinetic data show that TiO_2/Mmt has excellent antibacterial performance, and about 99% of both bacteria cells are inactivated within 75 min illumination. The AFM images demonstrate that the bacterial cells are irreversibly decomposed and some cell components are dissolved. Therefore, the content and phase of carbon and nitrogen in the solution are changed after photocatalytic reaction.

Key words: montmorillonite titanium dioxide; photocatalytic antibacterial; cell disruption; TOC/TN

1 Introduction

In 1985, Matsunaga and coworkers[1] reported for the first time the microbiocidal effect of TiO_2/Pt photocatalytic reactions. Since then, photo-induced antibacterial property of TiO_2 has been intensively investigated. In comparison with the conventional disinfection methods using freezing, radiation, dry-heat and chemical bactericide, TiO_2 has a broader spectrum and has been used in the photo-killing of bacteria, virus or phages[2, 3], cancer cells[4], algae[5], fungi as well as protozoan[6]. As to the bactericidal mechanism, there are different explanations[1, 7-11], which suggest that the main approach for photo-killing bacteria is as follows. When irradiated under ultraviolet (UV), TiO_2 generates electron-hole pairs and hydroxyl radicals, hydrogen peroxide, superoxide radical anions, *etc*. Then bio-chemical reactions occurs between these reactive oxygen species (ROS) and cell wall, cytoplasm membrane or intracellular components, causing damage to cell body or disorder in cell permeability resulting in the final cell death.

In this study, photo-killing experiments were conducted with both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria. Montmorillonite was used as a substrate to enhance the rate of photocatalytic reaction by adsorbing the reactants and enrich their concentration in the surface region of photocatalyst[12, 13]. The process of bacteria decomposition was investigated quantitatively by total organic carbon/total nitrogen (TOC/TN) analysis and qualitatively by atomic force microscopy (AFM) techniques. To our knowledge, there are few reports using montmorillonite supported titanium dioxide as photocatalytic bactericidal agent and few applications of TOC/TN analysis to follow the elemental changes in the process of reaction.

2 Experimental

2.1 Photocatalyst

TiO_2 loaded on montmorillonite was prepared by the sol-gel method. A solution of tetrabutyl titanate (TBOT) in ethanol (EtOH) was used as molecular precursor of TiO_2, and HNO_3 was used as a chemical additive to moderate the hydrolysis rate. The molar ratio of reactants was TBOT∶EtOH∶HNO_3∶H_2O = 1∶15∶3∶0.3,

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and the hydrolysis temperature was 25 ℃. Firstly, TBOT was dissolved into 2/3 of the total ethanol volume with stirring to make a solution named A, and the remaining 1/3 of ethanol was mixed with HNO_3 and deionized water to obtain a solution named B. Then B was gradually dripping into A under mechanical stirring. After B was completely added into A, keeping on stirring for 30 min and followed by ultrasonic dispersion for 10 min, a buff transparent sol was obtained. Secondly, a montmorillonite (Mmt for short) suspension was prepared. The mass concentration of Mmt was 3%, and the ratio of Ti(IV) to montmorillonite was 15 mmol/g. Then the sol was gradually dripped into the clay suspension under stirring. The final mixture was aged at 25 ℃ for 24 h, resulting in the formation of a white gel. The gel was then washed with deionized water, and dehydrated by centrifugation. The precipitate was dried in a vacuum oven at 60 ℃ and finally calcinated at 450 ℃ for 1 h to obtain the TiO_2/Mmt nano-composite.

2.2 Bacterial culture

Escherichia coli (ATCC 11229) and *Staphylococcus aureus* (ATCC 6538) were grown aerobically in nutrient broth medium(10 g peptone, 5 g beef extract and 5 g NaCl in 1000 mL distilled water) at 37 ℃ on a rotary shaker at 110 r/min. At the exponential or stationary growth phase, cells were collected by centrifugation at 5000 r/min for 5 min, washed twice with phosphate buffer saline (PBS, 0.03 mol/L, pH 7.2), and re-suspended in sterile deionized water (pH was adjusted to 7.2 using 1 mol/L NaOH). The initial cell concentration was determined with a biological microscope (Nikon YS100, Japan).

2.3 Photocatalytic reaction

Photocatalytic reaction experiments were carried out in a photochemical reactor (SGY-1, Nanjing StoneTech Electric Equipment Co. Ltd, China) as illustrated in Fig.1. Light from the middle-pressure mercury lamp (300 W) passed through a 365 nm band-pass filter. 10 mL bacteria suspension with about 4×10^5 colony forming units per milliliter (cfu/mL) was added into the quartz test tube. The photocatalyst dosage was 6 mg/mL. The photocatalyst-cell mixture was agitated continuously by air flow from a air-pump. The light intensity reaching the surface of the mixture measured by a UV radiometer (UVX, UVP Inc., USA) was 400 ± 10 μW/cm^2. Environmental temperature in the reactor was controlled at 25 ± 1 ℃ by two cooling-fans. Before experiments, photocatalyst and all vessels were sterilized by dry heat. Bacterial suspensions without TiO_2 were illuminated as a control, and the reaction of the photocatalyst-cell mixture in the dark was also carried out. Intermittent illumination experiment (illuminating for 45 min followed by an additional 30 min controlling in dark) was conducted to evaluate antibacterial property of TiO_2/Mmt after light was turned off. In the photocatalysis experiment, aliquots of 300 μL bacteria suspension were taken at 15 min intervals in 75 min for various analyses described below.

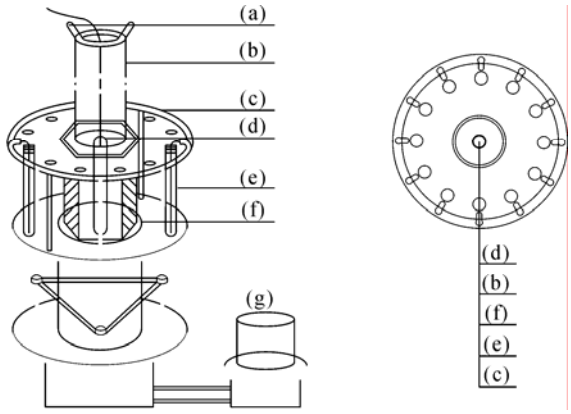

Fig.1 Schematic diagram of photocatalytic reactor. (a) inlet and outlet for cooling water; (b) quartz cold trap; (c) air distribution ducting; (d) middle-pressure mercury lamp; (e) quartz test tube; (f) light filter; (g) electromotor

2.4 Cell viability assay

The loss of cell viability was examined according to the viable count procedure. The viable count was performed by spreading 200 μL illuminated bacterial suspension onto nutrition agar plates in triplicates after serial dilutions in PBS. All plates were incubated at 37 ℃ for 24 h, then the colony units on the plates were counted.

2.5 Atomic force microscopy (AFM) imaging

Samples were analyzed using a digital atomic force microscope (DI Nanoscope IV SPM, USA) in contact mode. Before and after illumination, 10 μL of the illuminated bacterial suspension was dropped onto a mica sheet supported on a stainless steel plate (D=1 cm) and then air-dried for AFM imaging.

2.6 TOC/TN analysis

TOC (total organic carbon) and TN (total nitrogen) changes in the reaction solution were analyzed by a TOC/TN analyzer (Analytikjena, Muti N/C 2100, Germany). At each time interval, 100 μL of the illuminated bacterial suspension was diluted to 1 mL and centrifuged at 10 000 r/min for 5 min to precipitate solid materials. Then 100 μL of the supernatant was diluted to 5 mL for TOC/TN analysis.

3 Results and Discussion

3.1 XRD analysis of TiO_2/Mmt

The as-prepared TiO_2/Mmt photocatalyst were analyzed by X-ray diffraction (XRD) with a powder X-ray diffractometer (D/MAX-IIIA, Rigaku Co., Japan) to determine the crystalline phase of TiO_2. It shows that TiO_2 on the surface of montmorillonite is mainly anatase. The average size of TiO_2 particles calculated from XRD pattern is 9.8 nm by employing the Scherrer equation[14]: $D=k\lambda/\beta\cos\theta$, where, D is the diameter of the crystal; k the constant (k=0.9); λ the wavelength of the radiation used (λ=0.15405 nm, Cu Kα); β the full width at

half maximum (FWHM) of selected peak after correction for the instrumental broadening and θ the Bragg's angle of diffraction for the peak.

3.2 Effect of TiO_2/Mmt on inactivation of cells

E coli and *S aureus* cells were treated under different conditions, and the survival curves are shown in Fig.2. When the cell suspension with TiO_2/Mmt is controlled in dark, there is slight loss of cell viability for both bacteria (symbol △ in Fig.2). The metabolism effect of bacteria cells adsorbed by the substrate, or even the cells co-aggregation with photocatalyst would result in decrease of cell concentration[15] . It has been reported that montmorillonite has excellent adsorption capability for coliphages and reovirus[16], but the adsorption effect is relative to the electrokinetic potential of both clay particles and virus. On one hand, bacteria in aqueous suspension are always negatively charged[17]. On the other hand, the initial pH of bacteria cell suspension in this experiment is 7.2, close to the isoelectric point (IEP) range of the major commercial TiO_2 powders(IEP＝3-7.5[18], so TiO_2 surface should be neutral or negatively charged. Moreover, crystal lattice charge of montmorillonite renders it electronegative. The electrostatic repulsion between cells and photocatalyst make the adsorption effect on cells viability unremarkable.

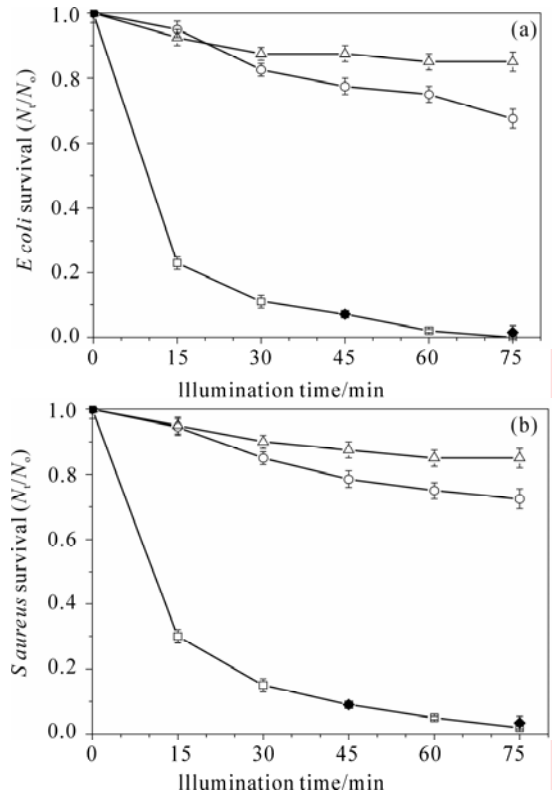

Fig.2 Effect of TiO_2/Mmt photocatalytic reaction on cell viability of *E coli* (a) and *S aureus* (b). Initial cell concentration: 4.0×10^5 cfu/mL for *E coli* and 3.5×10^5 cfu/mL for *S aureus*; photocatalyst dosage: 6 mg/mL; (□) Photocatalysis, UV+TiO_2/Mmt; (○) Photolysis, UV only; (△) Control in dark; and (◆) Intermittent illumination, illuminated for 45 min followed by an additional 30 min in dark.

However, the effect of cell adsorption on photocatalytic sterilization rate can not be ignored. As we know, in a heterogeneous catalytic reaction system, mass transfer is always the controlling factor, whereas adsorption of reactants on catalyst will conduce to mass transfer. Research[19] using TiO_2 immobilized on activated charcoal granules showed that the sterilization rate of *E coli* cells was remarkably enhanced through the ability of activated charcoal to adsorb the cells. Owing to the adsorption effect, in the first 15 min, the cell viability follows an exponential decay. While in the period of 15-75 min, the decrease in cell concentration assumes to be mild (Fig.2, symbol □), for dead bacteria and their excreted intracellular components would compete for photo-generated ROS and/or form a screen to the light penetration leading to the protection of the remaining active bacteria[20].

It is also observed that the killing rate is about 99% for both bacteria (decrease in cells concentration from 10^5 to 10^2-10^3). This impliys that TiO_2/Mmt photocatalyst hve excellent bactericidal performance. In comparison with photocatalyst, photolysis effect on bacteria inactivation is unremarkable (symbol ○ in Fig.2), for less hydroxyl radicals (·OH) are generated by UV irradiation. Generally, the wavelength of UV used in conventional disinfection method is 253.7 nm, shorter than the wavelength used in the experiment. In addition, the damage to bacteria cells is not always permanent, and this damage can be repaired through the enzymatic photo-reactivation and dark repair (or excision repair) mechanisms[20, 14].

Comparing (a) and (b) in Fig.2, It can be seen that both in the absence and in the presence of TiO_2/Mmt, the inactivation rate of *E coli* is a little higher than *S aureus*. This suggests that *E coli* exhibits a higher sensitivity to photolysis and photocatalysis than *S aureus*. It reveals that Gram-positive and Gram-negative bacteria respond differently to TiO_2 photocatalysis due to their structural differences, especially the complexity and thickness of the cell walls. This confirms the similar results obtained in previous studies[22, 6].

The kinetic data from Fig.2 (symbol ◆ and □) show that the viable cell count obtained at 75 min of the samples undergone intermittent illumination is close to the samples after continuous exposure to UV. It indicates that TiO_2/Mmt still exerts bacteriostatic effect on bacteria. The damage to cells in the dark continues via the Fenton reaction or the free radical chain reactions of lipid peroxidation[23]. This effect may be considered as a form of "residual disinfection effect[20].

3.3 Disruption of cells characterized by AFM

AFM has been proved to be a suitable method for investigation of cell morphology and structures[24]. Fig.3

illustrates the AFM images of *E coli* (a, b) and *S aureus* (c, d) at time zero and 75 min of photocatalytic treatment. Before the treatment, the intact *E coli* cells has a rod-like shape with a size of about 1 μm×1.5—2.5 μm (Fig.3a), while *S aureus* cells agglomerated together like a bunch of grapes with diameter of about 0.5 μm (Fig.3c). However, significant changes are observed after 75 min illumination in the presence of TiO_2/Mmt photocatalyst. The *E coli* cells are completely disrupted into pieces. The cell debris are dispersed in the suspension and the intracellular substances seem to be dissolved (Fig.3b). The damage to *S aureus* cells is not as severe as *E coli*, though the cell body shrank and is deformed, and there are fragments of the dead cells in the suspension (Fig.3d).

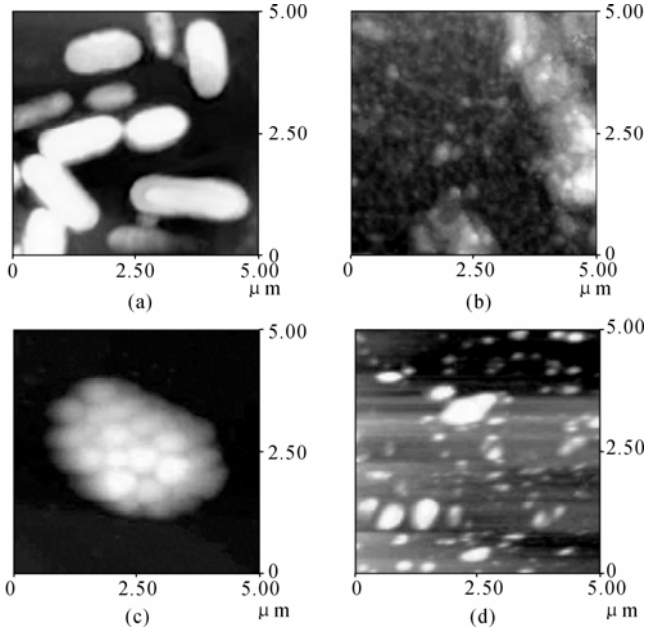

Fig.3 AFM images of the bacteria cells. *E coli* (a) and *S aureus* (c) before illumination; *E coli* (b) and *S aureus* (d) after illumination for 75 min

As mentioned previously, cell damage caused by photolysis is not persistent. However, AFM images indicate that the damage to bacterial cells by photocatalysis is irreversible. This provides the qualitative evidence for photocatalytic killing of cells.

3.4 Photocatalysis-related changes of TOC and TN

AFM studies indicates that the bacterial cells could be broken down by TiO_2/Mmt photocatalysis. Therefore, it is possible that the macromolecular components in bacteria, such as protein, polysaccharide and RNA, will be decomposed into small molecules and subsequently be dissolved. By assuming that the molecular formula for a bacterial cell was $C_5H_7NO_2$[25], the carbon and nitrogen content in cell body can be quantified. TOC/TN analysis was therefore used in the present work to examine the level of organic carbon and nitrogen (mainly Kjeldahl-N) dissolved from *E coli* and *S aureus*. The results are given in Fig. 4.

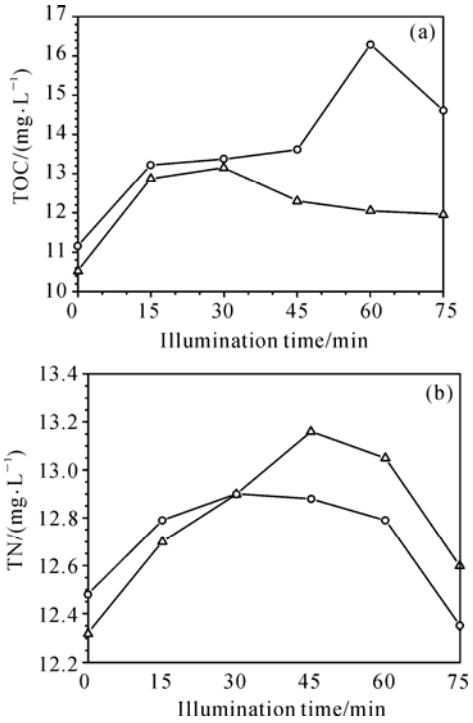

Fig.4 TOC (a) and TN (b) in reaction solution as a function of the illumination time. Initial cell concentration: 4.0×10^5 cfu/mL for *E coli* and 3.5×10^5 cfu/mL for *S aureus*; photocatalyst dosage: 6 mg/mL. (○) *E coli*; and (△) *S aureus*

For both bacteria, TOC and TN in the reaction solsution firstly increase and then decrease. The disruption of intact cells and decomposition of macromolecular components result in the transfer of carbon and nitrogen from solid phase to liquid phase. This corresponds to the increase in TOC and TN in the solution. With further illumination, the decrease in TOC and TN indicatea that the carbon and nitrogen are then transferred to gas phase by mineralizing into CO_2 and NH_3. This confirms earlier studies[26,28] that organic matters such as amino acids, DNA, RNA, *etc* are converted into NH_3, NO_3^- and CO_2 by TiO_2 photocatalysis.

Under the same experimental conditions, the quantities and rates of TOC and TN released from *E coli* and *S aureus* are different. The photocatalytic antibacterial mechanism proposed by previous researches[8,11]suggested that cell wall was the primary target attacked by ROS in the photo-killing process. These differences may mainly relate to the different structure and component of cell wall between these two types of bacteria. For *S aureus* ($Gram^+$), it has a thick peptidoglycan layer (20-80 nm) but no protein. Whereas, for *E coli* ($Gram^-$), the thickness of peptidoglycan layer is thinner (2-3 nm), and it has outer membrane protein[21]. Carbon exists extensively in each component of bacterial cell, while nitrogen element exists mainly in the peptidoglycan, amino acids, nucleic acids, protein and coenzymes. TOC and TN changes (Fig.4) during photocatalytic treatment reflect the mineralization extent of both cells. These data correlate well with the loss of cell viability (Fig.2), and quantitatively confirmed the ob-

servations from the AFM images (Fig.3). They also impliys that the inactivation of viable cells and the further decomposition of cell components occurs simultaneously.

4 Conclusion

From the above studies, montmorillonite supported titanium dioxide was proved to be an excellent photocatalytic antibacterial material for both *E coli* and *S aureus*. The electrostatic interactions between bacteria cells and photocatalyst have a significant effect on sterilization rate. Kinetics data from cell survival assay and TOC/TN analysis showed that Gram-negative bacteria seemed to be more sensitive to photocatalysis than Gram-positive ones due to their cell structural difference. The photocatalytic sterilization process was that cell inactivation followed by cell disruption and lysis leading to phase transfer of C and N elements in cell body.

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