Photocatalytic disinfection of *E. coli* using N-doped TiO_2 composite

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ABSTRACT

Heterogeneous photocatalysis is among an alternative technique for the inactivation of pathogenic micro-organisms. Several researchers have achieved the successful killing of bacteria, viruses, fungi or protozoa by semiconductor photocatalysis. This study significantly provides a better understanding of the bactericidal properties of N-TiO_2 by identifying specific bacterial targets and cell strcuture during disinfection in pure water. The photocatalytic inactivation of bacteria was investigated using *E. coli*, a well-known bacterial indicator. Firstly, the effects of the contact of N-doped TiO_2 with bacterial cells in the dark on both the bacterial cultivability and the envelope integrity was carried out. Then, assessment of the deleterious effects of N-doped TiO_2 on the bacteria's permeability and cultivability was done under visible radiations exposure. In order to identify the cell structure during the inactivation of the bacteria, monitoring of atomic force microscopy was also carried out.

Keywords: AFM, *E. coli*, inactivation, photocatalytic.

1 INTRODUCTION

Presence of potentially hazardous microorganisms and chemicals in water sources and lack of sanitation may cause severe health problems in human being. Because of this, still around one billion people do not get safe drinking water, and even millions of people die from waterborne diseases every year [1-3]. Water disinfection is a scientific and technical challenge since conventional methods used for drinking water supply such as chlorination, ozonation, and chloramination have shown disadvantages related to the formation of potentially hazardous disinfection by products (DBPs) [4, 5]. DBPs have been shown carcinogenic and mutagenic effects on mammals [5]. This led to severe criticism of its use in drinking water purification. Therefore, alternative methods of water disinfection have found great interest among researchers [1].

Heterogeneous photocatalysis is a promising alternative method for the inactivation of pathogenic microorganisms especially in sustainable energy and environmental purification area [6]. The heterogeneous photocatalysis consists of making use of the near-ultraviolet (UV) light (< 400 nm) to photo-excite a semiconductor catalyst in contact with water and in the presence of oxygen. As sensitizers, semiconductor particles (e.g., TiO_2) can act at room temperature. The photo-generated holes and electrons trapped on the surface of the semiconductor react with adsorbed species to initiate the formation of highly reactive oxygen species (ROS) capable of mineralizing pollutants. Two benefits of photocatalysis are its ability to damage microorganisms without further addition of chemical oxidant and secondly, the possibility of their use as a cost-effective and renewable source of energy. Now a days, many photocatalysts exist, such as ZrO_2, ZnO, Fe_2O_3, and WO_3, but TiO_2 is the most commonly used because of its chemical stability, low cost, low toxicity and high efficiency[1]. TiO_2-Photocatalytic inactivation of micro-organisms was first shown since 1985, Matsunaga et al. reported photokilling of Gram-negative, Gram-positive bacteria, yeast, and green algae. They observed that when TiO_2–Pt catalyst in contact with microbial cells was exposed to near ultraviolet light for 60–120 min, the cells in water could be killed. Concomitantly, it is widely used for the purification and disinfection of air and water, and the inactivation of prions, coccidian, bacteria spores, mycobacteria, viruses, fungi, yeast, and bacteria without spores [2, 7].

Development of new catalysts by inclusion of specific dopants reduce the band gap, and hence visible light may also be used to stimulate the photocatalytic activity, represents an important target. Its reactivity to light can be stretched to the visible region [7]. Noble metals (Ag, Cu, Pd), oxides (ZnO, WO_3, SiO_2) or non-metals (C, N, S, P) have been shown to broaden and enhanced the TiO_2 photocatalytic activity [8]. The doped films inactivated *E. coli* but the efficiency of the N,S-TiO_2 doped materials was an order magnitude below non-doped TiO_2 and therefore was not found suitable at present time for practical applications [7]. Highly oxidizing Ag, Cu metal nanoparticle can be used in conjunction with TiO_2 to attain a faster bacterial inactivation by hybrid-semiconductor materials. Bacterial cell surface is highly depending on environmental changes, more complex interactions can appear such as the association or dissociation of charged groups or bacterial conformational changes leading to either complexation with certain compounds, making the process more difficult to understand [9]. ROS exhibit bactericidal activity but some studies have emphasized that the hydroxyl radical would be the most important oxidant species responsible for the attack of the cell wall leading to bacterial inactivation. In addition, other papers reported the sensitivity of various microorganisms for photocatalysis: enveloped virus (HIV) > Gram-negative bacteria> Gram-positive bacteria > yeasts > fungi > enveloped virus >

mycobacteria > bacterial spores > coccidian > prions, when the structure and the complexity of the cell walls are considered [1, 2, 10]. The understanding of photo-disinfection kinetics and mechanism have significant importance for design, optimization and operation of large-scale photocatalytic water treatment processes. The bacteria inactivation processes can be observed that the inactivation profiles contain three different regions: (I) a shoulder region, (II) a log-linear inactivation region, and (III) a tail region [11-13]. The log-linear region covered most part of the bacterial inactivation reactions, the continuous perforation of the bacteria's outer membrane occurs [11]. The tail region is the part where the bacterial inactivation rate decreases at the end of the reaction. However, the mechanism of the tail region is still not well-understood.

Mechanistic models are more cumbersome but more robust than empirical models and are well suited for application to different situations such as the effects of photocatalyst loading, bacteria initial concentration, and light intensities. Langmuir-Hinshelwood-like kinetic equation was used, as a mechanistic model, for the description of the photocatalytic inactivation reaction rate. However, characterizing molecular interactions in the context of live bacteria represents a significant challenge. Unlike the conventional high-resolution electron microscope that must operate in vacuum, novel techniques of scanning probe microscopy, particularly atomic force microscopy (AFM), have a great potential for structural studies in microbiology.

2 MATERIALS AND METHODS

2.1 Materials

Synthesis of N-TiO_2 was carried out by using modified sol-gel method as reported by Lin *et al*[14]. First, ethanol, deionized water, and ammonium hydroxide, 200, 50, and 150 mL, respectively, were added to a flask placed into a 4°C water bath. The solution was stirred at 350 rpm and 10 mL of TTIP was added within 4 min. After 4 h of continuous stirring, the water bath cooler was turned off, stirring was maintained overnight. The mixture was centrifuged at 5000 rpm for 8 min. Resulting precipitate was obtained and washed twice using distilled water. Precipitated solids were dried in an oven at 105°C, overnight. Finally, N-TiO_2 powder was calcined in 500°C for 1 h [14]

2.2 Bacterial strains

The Gram-negative *Escherichia coli* was selected to carry out the photocatalytic experiments. The strains were bought from Bioresource Collection and Research Center (Food Industry Research and Development Institute, Taiwan). *E. coli* culture was prepared using nutrient agar and nutrient broth. Preparation for agars and broths were done by dissolving the required amount agar powder in the proper amount of deionized water. The powder was dissolved completely and was sterilized in an autoclave at 121°C for 15 min. Afterwards, if the solutions were not used immediately, these were stored at 5°C to 10°C. The corresponding amount of powder to be dissolved for each type of agar and broth are shown below. Prepare a liquid culture, 20 mL of broth was transferred to a sterile 250-mL Erlenmeyer flask.

2.3 Photocatalysis experiment method

The reactor was composed of a reactor box that enclosed the system. A cylindrical glass vessel (crystallizing dish) with a diameter of 13.5 cm and a height of 7.5 cm was used as the vessel for bacteria-catalyst reaction. Overhead visible light irradiation was provided by 9 pieces of white fluorescent lamps (8 W) with an intensity ranging from 6.76 to 7.23 mW/cm^2, and this was measured using a digital radiometer equipped with a 457 nm detector. Glass filters were used to cut off the UV light from the fluorescent lamps. Cooling fans were attached to both sides of the reactor to maintain a constant temperature. For control tests using TiO_2 Degussa P25 powder, UV-A irradiation was provided by 6 pieces of black light blue lamps (BLB lamps, 8 W, 365 nm peak emission) with a total light intensity of 1.88 mW/cm^2.

E. coli ATCC 8739 (BCRC 11634) was used to carry out the photocatalytic inactivation experiments. Prior to each experiment, an overnight liquid culture was prepared. From the liquid culture, bacterial cells were collected through centrifugation of 1 mL of the liquid culture at 8000 rpm for 10 min. Bacterial pellets were washed twice using sterile deionized water and then resuspended with 1 mL of sterile deionized water. Resuspended bacterial cells were diluted with sterile deionized water to obtain a 100-mL bacterial suspension with an initial concentration of 10^4 to 10^6 CFU/mL contained in the reaction vessel. The vessel was placed inside the reactor box. Catalyst powder, with concentration in the range of 0.6 to 1.0 mg/mL, was added to the suspension and stirred for 10 min in dark to reach equilibrium. The light source was switched on 80 min prior the experiment to stabilize the light intensity. The bacteria-catalyst suspension was then irradiated for 240 min with UV or visible light. The used catalyst was TiO_2 Degussa P25 or N-TiO_2, respectively. Stirring was maintained throughout the experiments. During the disinfection runs, the temperature was maintained constant at 20 ◦C±4 using two fans, and samples were collected periodically. At a regular time interval, 500-μL aliquot were obtained, diluted, and plated. Control experiments were performed without catalysts and another with catalysts in the dark. All agars, solutions, glassware, and pipette tips were sterilized in an autoclave at 121°C for 15 min before use. 500-μL aliquot obtained at a specified time interval and was serially diluted in sterilized deionized water. A standard serial dilution procedure was conducted, spreading 100 μL of each decimal dilution onto an agar plate (spread plate method).

The spread plating was done in triplicates. All plates were incubated at 37°C for 24 h in a temperature and humidity chamber (THC, 90% humidity). After incubation, the viable *E. coli* cells were estimated via colony-counting procedure and recorded as colony-forming-units per milliliter (CFU/mL). Cell concentration counts in the range of 30–300 colony forming units per plate were considered statistically significant.

2.4 Atomic force microscopy

In the AFM experiments, images were collected under different conditions. The *E. coli* inactivation experiments were conducted in different conditions such as catalyst dosage, bacteria concentration and different configurations with different reaction time were imaged. Control samples were not treated with the N-TiO_2. A 100 μL droplet of each test sample was applied onto a poly-L-lysine (PLL) coated glass slide and allowed to stand at 25 °C for 20 min. After deposition, the sample was rinsed 10 times with Milli-Q water, and air-dried at 25°C. On average, five individual bacterial cells were imaged at high resolution for each test. The experiments were performed in triplicate cultures for each parameter.

The AFM images were acquired using a Veeco D3100 (Berlin, Germany). Measurements were carried out in tapping mode using uncoated silicon XSC11/A1BS cantilevers from MikroMasch (NanoAndMore GmbH, Germany). XSC11/A1BS cantilevers had typical resonance frequencies of 350 kHz and a spring constant of 40 N/m. Height, error, and phase-shift images were recorded and images were line-fitted as required. Height and size information were acquired with the imaging software from NanoScope 6.0.

3 RESULTS AND DISCUSSION

The photocatalytic activity of nitrogen-doped TiO_2 was investigated through the inactivation of *E. coli* cells in suspension with the N-TiO_2 under visible light irradiation. Fig. 1 shows the bactericidal effect of 0.2 to 1.0 mg/mL N-TiO_2. In the control experiment, where bacterial suspension without catalyst was irradiated using visible light, there was an increase in the survival ratio. This indicates that the visible light had no significant photo-killing effect on the bacteria. The increase in survival ratio may be due to the presence of *E. coli* in the suspension that are in the process of cell division. Increasing the N-TiO_2 dosage in the suspension from 0.2 to 1.0 mg/mL increases the inactivation of *E. coli.* In this study, the optimum N-TiO_2 dosage was 1.0 mg/mL. A number of previous reports stated that increasing catalyst dosages beyond 1.0 mg/mL decreases the bactericidal effect. Due to this phenomenon, as the photocatalyst particles increase beyond the optimum dosage for bacterial inactivation, the particles tend to block portions of light which are entering into the bulk solution. This indicates the photons which are supposed to energize electrons in the photocatalysts are lessened and reducing the ROS generated, and decreasing the photocatalytic inactivation of bacteria [15]. After 240 min, 97.4% of 10^4 CFU/mL of E. coli was inactivated in the presence of 1.0 mg/mL of N-TiO_2 under visible light irradiation with intensity, 6.99 mW/cm^2. The effect of initial bacterial concentration was investigated by using 0.6-1.0 mg/mL of N-TiO_2 under visible light irradiation. As the initial bacterial concentration increases, the irradiation time also required to achieve the increase in total inactivation. If the initial bacterial density is lower, there are lesser bacterial cells that the photocatalyst particles need to interact with. Thus, complete inactivation of *E. coli* cells is reached faster.

In this research, the inactivation did not shows significantly delayed region for te begin of the experiment. Fig. 2 using Eq.1 modified chick-watson model has been use for fitting the result, and compared with Langmuir-Hinshelwood-like kinetic equation (Eq.2-3). Langmuir-Hinshelwood-like kinetic equation assume the kinetic constant (k), pseudo-adsorption constant (K) and inhibition coefficient (n) were constant in different stages [13].

Fig.3 shows the AFM images of *E. coli* cell dried in air, (a) is before the experiments, (b) and (c) are 30 and 180 min during photocatalystic inactivation. From the 3D images, it is clear that the membrane surface of the untreated bacterium is reasonably structured. A corrugated surface with no visible pores or ruptures was observed in all the examined cells. After photocatalytic inactivation (b) and (c), exposure of the cells to different dosage N-TiO_2 for 30 and 180 min led to greater membrane disruption. Some leaked contents and debris could also be detected around the partially disintegrated cells.

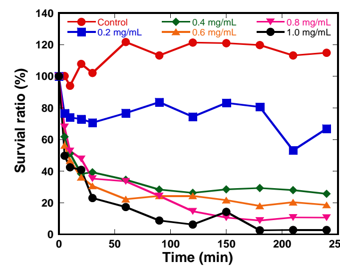


Figure 1. *E. coli.* survival ratio of inactivation using varying dosages of N-TiO_2, the initial concentration of cell was 10^4 CFU/mL .

Eq. 1 is modified Chick-Watson model and Langmuir-Hinshelwood-like kinetic equation show in Eq.2-3.

$$log\frac{N}{N_0} = k_1[1 - \exp(-k_2 t)] \quad (1)$$

$$\frac{dN_{undam}}{d_t} = -k\frac{KN_{undam}^n}{1 + KN_{undam}^n + KN_{dam}^n} \quad (2)$$

$$\frac{dN_{dam}}{d_t} = -k\frac{KN_{undam}^n - KN_{dam}^n}{1 + KN_{undam}^n + KN_{dam}^n} \quad (3)$$

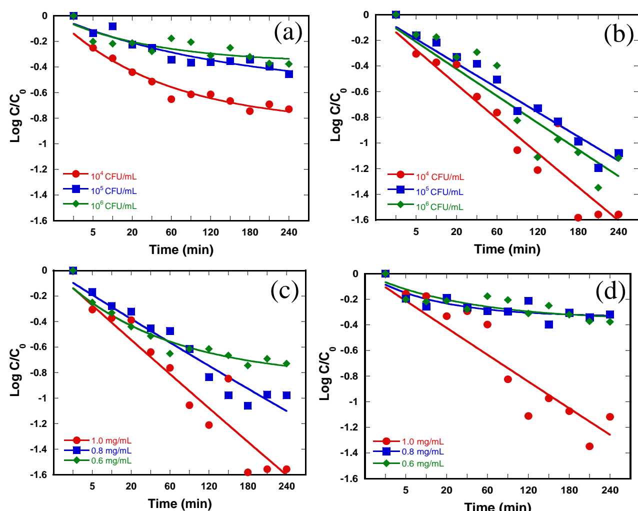
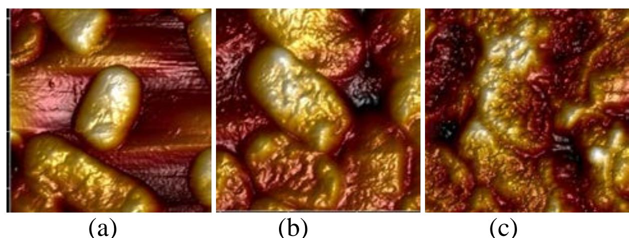


Figure 2. *E. coli.* ssurvival ratio of inactivation using varying dosages of N-TiO_2 and initial cell concentration under visible light and fitting with modified chick-watson model. (a) 10^4 and (b) 10^6 CFU/mL show initial cell concetntration effect; (c) 0.6 and (d) 1.0 mg/mL of N-TiO_2.



(a) (b) (c)

Figure 3. AFM images of E. coli cell dried in air. Before (a), 30 min (b), and 180 min (c) photocatalystic inactivation experiment were imaged (3 µm × 3 µm).

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