

CHARACTERIZATION AND BACTERIAL TOXICITY OF TITANIUM DIOXIDE NANOPARTICLES

Environmental Science

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ABSTRACT

India is a second highest populated developing country of the world. Environmental pollution is a big problem in India and the waste disposal laws are not as stringent as in the developed countries. Among the various nanoparticles, TiO_2 NPs are receiving increasing attention for a large variety of applications. Certainly, with the development of applications and production of the TiO_2 NPs, there is a major concern on the toxic effects due to their small size, large surface area and potential to produce ROS. TiO_2 NPs are highly mobile and reach the environment by numerous routes and get further degraded or modified into different forms by environmental factors. India carries a rich microbial diversity, producing industrial compounds like enzymes, antibiotic and biofertilizers. These massive number of nanoparticles continuously being released may lead to loss of our microbial diversity or effect their different physiological activities. In the present study, we showed how the structural differences in the cell walls of different bacteria play a very important role in the TiO_2 NPs toxicity which was assessed by traditional methods like growth inhibition assay as well as rapid dehydrogenase activity tests.

KEYWORDS

Environment, Pollution, Toxicity, Nanoparticles, Microbes, Titanium oxide

INTRODUCTION

Nanotechnology is a rapidly growing field of science and research that deals with extremely small particles ranging in size from 1 to 100 nanometers⁽¹⁾, contributing new materials with novel physical and chemical properties. Nanoparticles are being used in every sector like agriculture, medical, food sector and cosmetics^(2, 3). While nanoparticles have been found in environmental samples^(4, 5), the exact fate of these nanoparticles in the environment is still not identified. Nanoparticles like TiO_2 NP are gaining a lot of attention for their wide range of applications and their annual production is more than four million tons per year⁽⁶⁾. Toothpaste, dental fillings, photovoltaic cells, beauty products, sunscreens, and fabrics all contain TiO_2 NPs^(7, 8). Because it reflects and scatters ultraviolet (UVB and UVA) in sunlight, TiO_2 NPs is widely used in sun cream as a UV defender. TiO_2 absorb 70% of incident UV light and this leads to generation of hydroxyl ion and reactive oxygen species (ROS) in aqueous environment. This property of TiO_2 NPs is used in water treatment plants and in water purification systems. In sunscreens, TiO_2 NPs are either surface modified or embedded in the final product and therefore do not come into contact with consumer or environment, directly, however, after bathing and other day to day human activities, these nanoparticles reach the environment by diverse routes and are further degraded or modified to other forms by environmental factors. With the rising use of TiO_2 NPs at an ever-growing rate, there will undoubtedly be a large amount of TiO_2 NPs released into the environment, resulting in nanoparticle pollution. The small size of TiO_2 NPs contributes to their high mobility in the environment. The ecotoxicological impact of manufactured TiO_2 NPs and residues from commercial TiO_2 NP degradation is still not completely recognized. Microorganisms are present at the base of the food chain and play a chief role in maintaining an ecosystem. They are indispensable participants in numerous important elemental cycles in nature, including those of nitrogen, carbon, and sulfur. Microorganisms are the first living organisms to interact with xenobiotic compounds in the environment. Hence, they can be employed as a good model system, to assess the toxicity of TiO_2 NPs. Therefore, it is very important to check the toxic effects of TiO_2 NPs toward microorganisms like bacteria. Nanoparticles carry positive charge on their surface in solution, whereas microorganisms have negative charge on their surface. Consequently, there is a great likelihood of interaction between these two in the environment. Considering the above-mentioned reasons, it is extremely essential to assess the impact of new commercialized products, like TiO_2 NPs and residues of nanomaterials containing TiO_2 NPs on microorganisms and at a larger scale on the ecosystem.

MATERIALS AND METHODS

Preparation of TiO_2 NPs stock suspension:

TiO_2 nano-powder Aeroxide P25 was purchased from Sigma-Aldrich. These nanoparticles are widely used as photocatalysts and have been well investigated. Aeroxide P25 is an 80:20 anatase: rutile mixture with a primary particle size of about 21 nm and specific surface area of 35-65 m^2/g . The suspension was prepared by dispersing 100 mg of

TiO_2 NPs in 10 ml sterile ultrapure water (milli-Q water). The resultant suspension was then probe-sonicated (Sonics Vibra-cell 750W, Sonics & Materials, frequency 20 kHz, 3 mm micro tip, amplitude 40%) for 20 min at 4°C to homogenize and break apart the larger agglomerates.

Physicochemical Characterization Of Nanomaterials:

The shapes and primary sizes of nanoparticles were determined by TEM using a TECNAI 200 kV TEM (Fei, Electron Optics) at electron microscopy facility, AIIMS, Delhi. Samples were prepared by evaporating a droplet of nanoparticle suspension on a copper grid at room temperature. Size was determined using 100 randomly chosen nanoparticles. DLS measurements were employed to ascertain the hydrodynamic diameter of the nanoparticles. Investigations on the electrophoretic mobility of the nanoparticles were done to determine the zeta-potential. Both studies were conducted with a Zetasizer equipment (Nano ZS Malvern Instruments, UK). Measurements were made on colloidal nanoparticle suspensions in milli-Q water.

Preparation of Resting Cells:

Laboratory strains of *Escherichia coli* MG1655, *Pseudomonas aeruginosa* A01, *Salmonella enterica*, *Agrobacterium tumefaciens* IP-671, *Sphingomonas* sp. and *Corynebacterium glutamicum* Res 167 were used for this study. Cells were inoculated in 100 ml of a Luria-Bertani broth and then incubated at 37°C at 150 rpm until exponential phase of growth was attained ($\text{OD}_{600 \text{ nm}}$: 0.5). The cells were next harvested by centrifugation at $8000 \times g$ for 10 min and washed twice with sterile milli-Q water (pH 6). The bacterial suspension obtained was adjusted to an $\text{OD}_{600 \text{ nm}}$ of 0.4 (10^9 cells/ml) and used immediately for assays.

Toxicity Assessment:

Toxicity of TiO_2 NPs toward the bacterial strains was investigated using the growth curve inhibition, dehydrogenase assay and BacLight bacterial viability kit.

Toxicity Assessment By Growth Curve Inhibition:

To study toxicity of TiO_2 NPs toward the bacterial strains 500 μl of TiO_2 NPs exposed or unexposed cells were transferred to 250 ml Erlenmeyer flasks containing 50 ml of Luria-Bertani broth for bacterial growth inhibition in liquid media. The flasks were then kept at 37°C on a rotary shaker (150 rpm) and the $\text{OD}_{600 \text{ nm}}$ was measured every hour to monitor the bacterial growth till 20 hrs. By comparing the absorbances of TiO_2 NP exposed and undamaged cells percentage of viability was calculated.

Toxicity Assessment By Dehydrogenase Activity Assay:

Toxicity assessment was done using a rapid and accurate dehydrogenase assay. This assay is generally used as indicators of cell redox activity in order to test resistance to solvents, drug susceptibility or specific substrate utilization. In this study, the conventional dehydrogenase assay was standardized to assess the toxicity of TiO_2 NPs towards the already well studied strain of *E. coli* and then was

used to assess toxicity for other bacterial strains. The respiration was measured using 150 ml reaction mixture in each 96 well microtiter plates. Reaction mixture was prepared using M9 medium, TiO₂NPs exposed cells, 10 mM NaCl and 0.05 % 2, 3, 5-Triphenyl tetrazolium chloride (TTC) reagent. Microtiter plate were then incubated at 30°C on shaking (200 rpm). The amount of TTC conversion by TiO₂NPs exposed cells and unexposed cells was measured using Microtiter Plate Reader at 480 nm after every hour till 20 hrs.

Toxicity Assessment By BacLight Bacterial Viability Kit:

The Live/Dead BacLight bacterial viability kit (Invitrogen) was used according to the manufacturer's instructions. This kit employs SYTO 9 and Propidium iodide to differentiate cells with intact (live organisms-stained in green) and damaged (dead organisms-stained in red) membranes. BacLight-stained samples were viewed using an epifluorescence microscope (Nikon ECLIPSE, TS100 and Type 120) with the appropriate filter cube. The percentage of viable cells was determined by comparing the number of red and green cells obtained with the exposed and unexposed samples.

RESULTS AND DISCUSSION

Size characterization of the TiO₂NPs stock suspension:

DLS measurements revealed that the average hydrodynamic diameter of the dispersion decreased significantly, and became relatively constant after 30 min. The hydrodynamic diameter obtained (80-1000 nm, average size: 220 nm) was larger than the primary particle sizes obtained by TEM (20-90 nm, average size: 26 nm) and given by the manufacturer (average size: 21 nm). These findings suggest that the TiO₂NPs stock suspension is largely composed of small aggregates (Fig. 1). Zeta potential of TiO₂NPs in MQ was 25 mv. TiO₂NPs were positively charged in MQ water. TiO₂NPs were found to make aggregates in milli-Q water as shown in Figure 1.

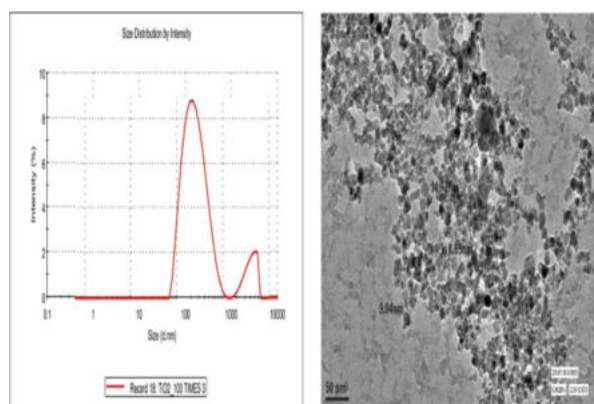


Figure 1: Physical characterization of the TiO₂NPs stock suspension. Particle size distribution by DLS measurement and transmission electron microscopy image of the TiO₂NPs after 20 mins of sonication in MQ water.

Toxicity Assessment:

The dose-dependent effect of TiO₂NPs on bacterial strains belonging to different genus, *P. aeruginosa* A01, *S. enterica*, *A. tumefaciens* IP-671, *Sphingomonas* sp., *C. glutamicum* Res 167 and a sensitive *E. coli* MG1655 strain was studied using growth curve inhibition, dehydrogenase activity test.

Out of these bacterial strains the Gram-negative strains; *P. aeruginosa* A01, *S. enterica*, *A. tumefaciens* IP-671, *Sphingomonas* sp. were found to be sensitive to TiO₂NPs as in the case of sensitive *E. coli* MG1655 strain (Figure 2 a and b). Whereas, the Gram-positive strain, *C. glutamicum* Res 167 strain was found to be resistant to TiO₂NPs.

Cell viability for *E. coli* MG1655 was found to be 16% in 100 mg/l TiO₂NPs sample after 20 hrs exposure by both growth curve inhibition and dehydrogenase assays.

Similarly, the cell viability for *S. enterica*, *A. tumefaciens* IP-671, *Sphingomonas* sp., *P. aeruginosa* A01 and *C. glutamicum* Res 167 was found to be 31%, 38%, 24%, 45% and 98% in 100 mg/l TiO₂NPs samples after 20 hrs exposure by both growth curve inhibition and dehydrogenase assays, respectively.

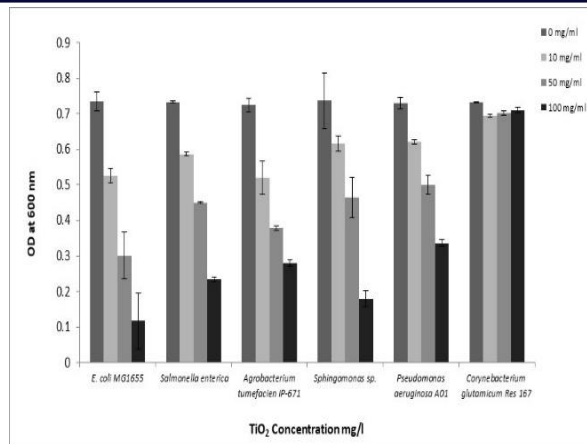


Figure 2 (a): Cell viability after 20 hrs of exposure to different concentration of TiO₂NP assessed by growth curve inhibition

The toxicity of TiO₂NPs was found to be dependent on their concentration; therefore, the described microplate dehydrogenase activity assay could be used as a rapid and sensitive method for detection of bacterial growth inhibition. Reading obtained by Live/Dead BacLight bacterial viability assay taken after 0 hr and 20 hrs for TiO₂NPs exposed and unexposed samples also showed similar viability results.

In this study, TiO₂NPs toxicity towards bacterial strains was found to be concentration dependent. The low cell viability of the Gram-negative strains; *E. coli* MG1655, *S. enterica*, *A. tumefaciens* IP-671, *Sphingomonas* sp. and *P. aeruginosa* A01 could be due their thin cell wall layer which enables the entry of ions released from TiO₂NPs into their cell. Also, as lipopolysaccharide (LPS) molecules carry a negative charge, they have a higher affinity for the positively charged TiO₂NPs. Which ultimately damage the essential proteins or biomolecules on the cell wall surface. The large aggregates of bacteria and TiO₂NPs were clearly visible by naked eyes at the bottom of the flasks during the growth inhibition experiment. This evidently show that TiO₂NPs get adsorbed on the bacterial surface and impart their toxic effects. This aggregation of nanoparticles with bacteria have also been reported in many studies⁽⁹⁾.

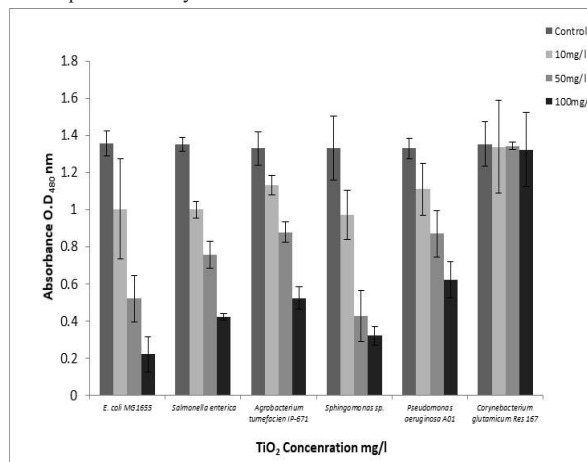


Figure 2 (b): Cell viability after 20 hrs of exposure to different concentration of TiO₂NP assessed by dehydrogenase activity assay

The reported mechanism of TiO₂NPs toxicity is cell membrane damage and leakage of the intracellular content. Some bacteria are resistant to TiO₂NPs, however TiO₂NPs nevertheless attach to the surface of these bacteria⁽¹⁰⁾. *C. glutamicum* Res 167, a Gram-positive bacterium was found to be resistant to TiO₂NPs toxicity because it has a much thicker protective cell wall (~20-80 nm) composed of peptidoglycans and covalently bonded teichoic acids. Although, Gram-negative bacteria like *Cupriavidus metallidurans* has been reported to be resistant to nanoparticle toxicity, indicating the role of upregulation of crucial genes acquired by horizontal gene transfer, the underlying mechanism is still not known^(11, 12). Several studies on

nanoparticles toxicity have reported very low or no toxicity of nanoparticles towards Gram-positive bacteria^(13, 14). Apart from this, toxicological effects of NPs are also reported to be influenced by several parameters, like pH, ionic strength, cations, natural organic matter^(15, 16, 17). Any minor change in any of these factors alters nanoparticles size, surface area and zeta potential dramatically and consequently change their toxicity levels. Therefore, these factors should be clearly monitored during the toxicity evaluation studies. Moreover, further studies are required to assess the toxic effect of TiO₂NPs towards Gram-positive bacteria at molecular level along with the impact of environmental condition to completely understand the mechanism involved in the TiO₂NP toxicity towards bacteria.

CONCLUSION

The findings revealed that TiO₂NPs toxicity was concentration dependent, resulting in a drop in growth rate and cell death. Also, TiO₂NPs toxicity is determined by the physio-chemical properties of nanoparticles and the morphological characteristics of the bacteria like cell wall peptidoglycan which stabilize the cell wall layers and facilitates the efflux of membrane penetrating TiO₂NPs. To further understand the exact molecular mechanisms of toxicological effects of TiO₂NPs, bacterial mutants with different surface properties can be explored.

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