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Cytotoxicity of TiO_2 nanoparticles towards freshwater sediment microorganisms at low exposure concentrations

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ABSTRACT

There is a persistent need to assess the effects of TiO_2 nanoparticles on the aquatic ecosystem owing to their increasing usage in consumer products and risk of environmental release. The current study is focused on TiO_2 nanoparticle-induced acute toxicity at sub-ppm level (≤ 1 ppm) on the three different freshwater sediment bacterial isolates and their consortium under two different irradiation (visible light and dark) conditions. The consortium of the bacterial isolates was found to be less affected by the exposure to the nanoparticles compared to the individual cells. The oxidative stress contributed considerably towards the cytotoxicity under both light and dark conditions. A statistically significant increase in membrane permeability was noted under the dark conditions as compared to the light conditions. The optical and fluorescence microscopic images showed aggregation and chain formation of the bacterial cells, when exposed to the nanoparticles. The electron microscopic (SEM, TEM) observations suggested considerable damage of cells and bio-uptake of nanoparticles. The exopolysaccrides (EPS) production and biofilm formation were noted to increase in the presence of the nanoparticles, and expression of the key genes involved in biofilm formation was studied by RT-PCR.

1. Introduction

Titanium dioxide nanoparticles (TiO_2 NPs) have a broad range of commercial applications, especially in consumer products (Newman et al., 2009). Nanotechnology-based products are estimated to achieve $3 trillion market with six million workers by 2020 (Liu et al., 2013). Owing to the sharp increase in TiO_2 NP usage in industry, an increase in their release into the environment along with industrial effluents is also anticipated. It has been reported that TiO_2 NPs can enter the aquatic environment either through direct or indirect release from nano-paints, sunscreen lotions, food additives, medical use, dismantling of batteries, recycling of plastic/glass/metal with nano-coating, and groundwater remediation (O'Brien and Cummins, 2010), which in turn may cause toxicity to the living organisms in the environment.

The presence of microorganisms is essential for maintenance of a sustainable ecosystem. Microorganisms being the chief agents of early transformation of organic matter, regeneration of nutrients as well as food source for higher trophic level play an important role in ecosystem. Bacteria are being increasingly used as possible test systems for evaluating nanoparticles toxicity. Not only they are deemed as receptors necessary to study ecological nanotoxicology (Holden et al., 2012), but they also act as facile test subjects that can be used in miniaturized toxicological screening for rapid hazard identification (Jin et al., 2010; Nel et al., 2013). The conclusions from the bacterial toxicity assay can encourage the development of safer nanomaterial designs (Wang et al., 2012).

From a thorough literature review, the interaction between the nanoparticles and the bacterial cell leading to cytotoxicity has been hypothesized to involve two steps. The first step is the oxidative damage by the nanoparticles attached to the cell membrane, resulting in loss in membrane integrity without significant reduction in cell viability. The second step involves leaking out of intracellular components, which is the leading cause

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of decreased viability and internalization of the nanoparticles, thereby causing damage to cell organelles including the nucleus (Klaine et al., 2008). Previous reports suggest that the adsorption of TiO_2 NPs onto the cell wall, interruption of the transmembrane electron transfer, modification of the membrane potential, physical damage resulting in leakage of the cell contents, and the production of reactive oxygen species (ROS) were among the major contributors in the cell–nanoparticles cytotoxic interactions (Klaine et al., 2008; Li et al., 2008).

Generally the concentration of TiO_2 NPs in various environmental compartments across several continents has been found to be in the range of 0.7–16 μg/L (Mueller and Nowack, 2008). The cytotoxic effects of the nanoparticles may be strongly influenced by their aggregation/agglomeration in the test system, with the smaller/more stable particles having enhanced penetration and accumulation in the cells, causing more damage of the cells than that of larger/agglomerated particles (Navarro et al., 2008). Since there is evidence showing free radical generation by TiO_2 NPs under both visible light and dark conditions (Sayes et al., 2006; Lipovsky et al., 2012) cytotoxicity under differing irradiation conditions requires attention. These influencing factors need to be kept in mind while designing a complete toxicity assay involving environmental microorganisms and nanomaterials.

Most of the prior reports regarding environmental toxicity of TiO_2 NPs dealt with individual bacterial cells, which may not always be the case in the natural environment. It can be hypothesized that the consortium being heterogeneous in nature may experience less toxic impact of the nanomaterial in the environment than the individual bacterial cells, which are homogenous. The aim of the present study was to elucidate the phototoxic effects of TiO_2 NPs to different individual bacterial species isolated from the freshwater sediments as well as their consortium (a mixture of a number of different cultures) at low exposure concentrations (0.25, 0.50, 0.75 and 1.00 μg/mL) in a freshwater system without any nutrient supplements (to mimic the chemical matrix of a freshwater aquatic environment) under visible light and dark conditions.

2. Materials and methods

2.1. Materials

Titanium oxide NPs were procured from Sigma Aldrich (dry titanium (IV) dioxide nanopowder, 99.7% anatase, CAS no.: 637254). Ethidium bromide (EtBr) was procured from Medox Biotech India Pvt. Ltd. Acridine Orange (AO) was obtained from Hi-Media Pvt. Ltd. (Mumbai, India). 2′,7′-Dichloro fluorescein diacetate (DCFH-DA) was purchased from Sigma Aldrich (St. Louis, MO, USA). All other chemicals used throughout the experiment were of analytical reagent grade.

Lake water from VIT Lake, Vellore, India, was collected (12°58′ 10″N, 79°937″E) (Pakrashi et al., 2013). ICP-OES (Perkin Elmer Optima 5300 DV, USA) was carried out in order to quantify the metal ions (Al^{3+},Cu^{2+}, Zn^{2+}, Mn^{2+}) and other inorganic ions (SO_4^{2-}, PO_4^{3-}, NO^{3-}, Cl^-) present in lake water by titrimetric methods (details are provided in our previous report) (Pakrashi et al., 2011). Lake water was filtered through Whattman No. 1 filter paper to remove suspended dust particles, followed by sterilization keeping the chemical composition of the water unperturbed. A secondary filtration, through a 0.22-μm membrane filter, was done to avoid the interference of large colloidal particles prior to hydrodynamic size measurement (Pakrashi et al., 2011). The sterilized lake water was used as the experimental matrix throughout the study (Dalai et al., 2014).

2.2. Stability analysis of nanoparticles dispersion

To study the colloidal stability of TiO_2 NPs in the lake water at the time periods of 0, 2, 4, 6, and 24 h mean hydrodynamic size analysis by dynamic light scattering was carried out using 90 plus Particle Size Analyzer, Brookhaven Instruments Corporations, USA. A stock dispersion of TiO_2 NPs (100 μg/mL) was prepared in MilliQ water and sonicated using an ultrasonic processor (Sonics, USA) of 350 W for 10 min. A working concentration of 1 μg/mL of TiO_2 NPs was prepared in filtered lake water by diluting the required volume of stock solution. Hydrodynamic size of the particles in the suspension was measured at time intervals of 0 h, 2 h, 4 h, 6 h and 24 h by dynamic light scattering method using a 90 plus Particle Size Analyzer (Brookhaven Instruments Corporations, USA) (Dalai et al., 2012).

The actual concentration of TiO_2 NPs in the lake water medium was measured by ICP-OES (Perkin Elmer Optima 5300 DV, USA). Four different concentrations, i.e., 0.25, 0.50, 0.75, and 1 μg/mL, were prepared by adding desired volume of stock in 20 mL working volume of lake water. The samples were then acid digested and analysed by ICP-OES (Perkin-Elmer Optima 5300 DV, USA).

2.3. Isolation and identification of bacteria

Sediment sample from the VIT Lake, VIT University, Vellore, India (conductance: 4.3 ± 0.13 mS cm^{-1}; pH: 7.8; DO: 7.2 ± 0.46 μg/mL; TDS: 800 $\pm$ 74 μg/mL) was collected and stored in polypropylene bottles at 4 °C used for microbiological analysis. 1 g of sediment sample as added to 100 mL of sterile water, stirred vigorously and then allowed to sit for the soil to settle. Serial dilution of the sample was carried out with sterile distilled water. This suspension was added to a nutrient agar plate and kept in an incubator at 30–37 °C. After 24 h the observed bacterial colonies with different colony morphologies were isolated and labelled. The isolates were then plated again on a nutrient agar medium to obtain pure cultures.

2.4. Development of the ternary consortium

To examine the toxic effects of TiO_2 NPs, three individual bacterial species and a bacterial consortium (a mixture of cultures) were prepared. Antagonistic/synergistic studies were carried out with three bacterial isolates (*Bacillus alitudinis*, *Bacillus subtilis* and *Pseudomonas aeruginosa*) for the development of the ternary consortium. One isolate (*B. alitudinis*) was taken and grown in nutrient broth, after 4 h of incubation; 100 μL of culture broth was poured onto the surface of nutrient agar and a loop full of culture of the second isolate (*B. subtilis*) was streaked in the middle of the plate. Same step was followed in case of *B. subtilis*, *P. aeruginosa* and *B. alitudinis*, *P. aeruginosa*. After that the plates were incubated at 30–37 °C for 24 h, and then the plate was inspected. The zone of inhibition between the isolates was absent, which showed that they lacked competitive inhibition (Samuel et al., 2012). With the developed ternary consortium, toxicity study of TiO_2 NPs was carried out.

2.5. Toxicity assessment

2.5.1. Experimental setup

The bacterial isolates from fresh water sediment (VIT lake water, Vellore, India) were identified as *B. alitudinis* (gram positive rods), *B. subtilis* (gram positive rods), and *P. aeruginosa* (gram negative rods) through 16S rRNA analysis (length of the sequence, 1462 bp; homology, 99%).