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Cytotoxicity of titania nanoparticles towards waste water isolate *Exiguobacterium acetylicum* under UVA, visible light and dark conditions

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ABSTRACT

The aim of the current study was to explore the different modes of the cytotoxicity effects of titania nanoparticles (TiO_2 NPs) towards the dominant waste water isolate *Exiguobacterium acetylicum* at an exposure dose of $\leq$1 μg/mL in the sterilized and filtered waste water already containing 0.770 ± 0.02 μg/mL dissolved Ti^{4+} ions, under different conditions (UVA, Visible Light and Dark). The aggregation behaviour of TiO_2 NPs in the experimental matrix was studied for 0.25, 0.5 and 1 μg/mL concentrations at different time intervals (0, 6 and 12 h). The reduction in cell viability under UVA was significantly more than that of other two conditions, i.e., dark and visible light. At 1 μg/mL of titania NPs for an exposure period of 6 h the cell viability with respect to control was found to be as follows: UVA (11.1 ± 0.33%) <visible light (83.8 ± 1.3%) <dark (84 ± 1.3%). The generation of oxidative stress (hydroxyl and superoxide radicals) was more in the bacterial cells exposed to UVA condition as compared to the cells exposed to visible light and dark condition. A significant increase in the membrane permeability was noted under UVA conditions in comparison to that in dark and visible light conditions. The fluorescence microscopy confirmed cell damage due to exposure to the nanoparticles. The scanning electron microscopy (SEM) analysis demonstrated the morphological distortion in the nanoparticle treated bacterial cells. The adsorption and further internalization of nanoparticles were confirmed by transmission electron microscopy (TEM) and elemental analysis.

Introduction

Manufactured TiO_2 NPs are being continuously used in the commercial and consumer products like sunscreens, paints, cosmetics, surface coatings, medical use, food additives, dismantling of batteries, recycling of plastic/glass/metal with nano-coating, groundwater remediation etc. Hence, it enhances the potential risk of the nanoparticles entering into the aquatic environment [1]. The expanding commercial usage of the particles has led to the ambiguity regarding use and disposal of nanoparticles into the environment, and the released nanoparticles finally ends up in the waste water [2]. The concentration of a nanomaterial in the waste water is dependent on various factors: (a) The amount of nanomaterial produced; (b) The amount of nanomaterial that reaches the waste water stream and the degree of dilution; (c) The amount of 'fixed' and 'free' nanomaterial in the commercial product; (d) The extent of agglomeration or adsorption occurring in waste water streams that changes the form of the nanoparticle or move it out from solution. Q3

The draft permissible exposure limit (PEL) for TiO_2 NPs proposed by the National Institute for Occupational Health and safety (NIOSH) has been set to 1.5 mg m^{-3} and a recommended exposure level (REL) has been set at 0.1 mg/m^3 [3]. However, the concentration of titania NPs in waste water treatment plants as reported was around 185 μg/L [4]. In another study Ti concentration in the waste water treatment plant was found to be in a range of 181–1233 μg/L [5]. The effluents released from waste water treatment plants and waste incineration of products was observed to be the primary entry points of TiO_2 NPs to the aquatic environment.

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Bacteria being the key factor of food-chain play an essential role in the maintenance of the ecosystem. Any adverse effect on the bacterial population can cause destructive effects on the ecosystem [6]. The presence of titania NPs in the water affects the microbial community that in turn leads to deleterious effects on the environment [7]. The interactions of manufactured nanoparticles and bacteria may lead to the alteration of bacterial morphology leading to partitioning, changes in the nutrient cycling and trophic transfer to higher organisms [8]. The activity of TiO_2 NPs in the environment relies on the colloidal stability of the nanoparticles and the increment in the diameter of the particles [9]. Nano titania is predominantly photocatalytic in nature [10]. According to a report, 100% mortality of *Escherichia coli* with a cell number of 10^6 was observed due to the exposure of the cells to 100–1000 μg/mL of titanium dioxide with photocatalytic UV conditions [11]. The reduction of bacterial cell viability was possible even in dark condition demonstrating the possibility of non-photocatalytic mechanism [12]. It has been reported that the TiO_2 NPs can induce the oxidative stress in bacteria, which plays a significant role towards its toxicity [13]. The ROS production results in oxidative stress in the micro-organisms, leading to cell death [14].

The present study has been carried out to demonstrate the interaction between TiO_2 NPs and *Exiguobacterium acetylicum* (isolated from VIT sewage treatment plant, Vellore, Tamil Nadu, India) under UVA, visible light and dark conditions in the waste water matrix itself. It can be hypothesized that TiO_2 NPs being photocatalytic in nature would cause more cytotoxicity on *Exiguobacterium acetylicum* under UVA than that of visible light and dark condition. UV radiation can penetrate to a considerable depth of aquatic eco-system. Based on the wavelength there are three types of UV radiations; UVA, UVB and UVC. UVA is the longest wavelength ranging from 320 to 400 nm that comes from the sunlight directly and reaches the surface of the earth [15]. Considering the fact that TiO_2 NPs are photocatalytic in nature UVA irradiation was selected as photo-condition for the present study. The predicted environmental concentration of TiO_2 NPs was computed at 1 μg/L [16]. In addition, the type of environmental matrix may play a significant role in controlling the aggregation behaviour of the nanoparticles, and thus may modulate their toxic effects [17]. To mimic the environmental conditions filtered and sterilized waste water already containing dissolved Ti^{4+} ions was selected as the experimental matrix for the overall study, with added TiO_2 NPs at low exposure concentrations ($\leq$1 μg/mL). To the best of our knowledge, the present study is first of its kind that deals with the interactions of TiO_2 NPs with waste water bacterial isolate which was already exposed to Ti^{4+} ions, in a medium mimicking the chemical conditions prevalent in the waste water. The study also emphasizes on the comparisons of the environmental relevant conditions like UVA, visible light and dark.

Materials and methods

Titanium dioxide (IV) nanoparticle anatase (99.7%) with a particle size <25 nm, CAS no: 637,254 was obtained from Sigma–Aldrich (St. Louis, USA) suppliers. 2-[6-(4′-amino) phenoxy-3H-xanthen-3-on-9-yl] benzoic acid (APF), DHE (Dihydroethidium) fluorescence probes were procured from Life Technologies. Acridine Orange (AO) was obtained from Himedia Pvt. Ltd., and Ethidium Bromide from Medox India Pvt. Ltd. All the chemicals used were of analytical grade.

The waste water samples were collected in sterile polypropylene bottles from VIT sewage treatment Plant (Secondary treatment tank), Vellore, India. The primary filtration was carried out with the help of 20 μm sieve to remove the solid particles further followed by Whatman No. 1 filter paper and secondary filtration with 0.22 μm filter to elude intrusion with the colloidal particles. The filtrate samples were autoclaved and stored in a sterile screw-capped bottle. The waste water used for the experiments exhibited a pH of 7.5, dissolved oxygen, 5.38 mg/L, total dissolved salts, 1.14 ± 0.05 μg/mL, conductance 161 ± 0.88 mS, total organic carbon 16.16 ± 0.59 μg/mL, inorganic carbon 33.7 ± 0.02 μg/mL. The parameters were determined following the American Public Health Association protocols [18]. The metal ions present in the waste water were quantified by ICP-OES (PerkinElmer optima, 5300, USA) (Table S1 supporting material). The waste water medium contained 0.770 ± 0.02 μg/mL dissolved Ti^{4+} ions prior to addition of TiO_2 nanoparticles. The filtrate waste water was used as a natural matrix for bacteria and titania nanoparticle interaction studies.

Particle size determination of titania nanoparticles in the test medium

A stock suspension of Titanium dioxide (TiO_2) nanoparticle was prepared for 100 μg/mL in Milli Q water and subjected to ultrasonication at 130 W for 10 min. (Ultrasonics, USA). The working concentrations of 0.25, 0.5 and 1 μg/mL were prepared by adding the appropriate amount of stock dispersion in the waste water under UVA and visible light conditions. The effective diameters of the particles were measured using 90 plus particle size analyser (Brookhaven Instruments corporations, USA).

Isolation and identification of the isolate

The cultures were isolated from the waste water samples collected from VIT Sewage treatment Plant, Vellore, India and were serially diluted using aseptic techniques. The isolation was primarily carried out by streaking the cultures on a nutrient agar plate and incubating at 37 °C for 24 h. The isolated colonies were subcultured to obtain a pure culture [19]. Morphological characterization, Gram staining was performed, and the isolates were submitted for 16S-rRNA characterization.

The morphology of the dominant bacterial isolate was found to be circular, rod shaped. The molecular characterization was executed for the isolate and was found to be *Exiguobacterium acetylicum* (VIT WW 1). The dominant waste water isolate showed 99% similarity in BLAST search to the corresponding sequences available and the length of the sequence was estimated to be 818 bp. The bacterial genomic DNA was extracted using phenol-chloroform and fluorescent dye terminator method (ABI Prism, Big dye terminator cycle sequencing kit 3.1) [20]. Sequences were scrutinized with BLAST and aligned with the help of CLUSTAL W utilizing the sequences phylogenetic tree was constructed using neighbour joining method. The 16SrRNA sequence of the strain was submitted to Genbank, and the accession ID (KJ146070) was obtained.

Cell viability

A stock of 100 μg/mL titania nanoparticle was prepared in filtered and sterilized waste water. The lower exposure concentrations of 0.25, 0.5, 1 μg/mL was prepared by diluting the stock suspension. An initial bacterial count of 5×10^8 CFU/mL was selected for the overall studies. Three conditions namely, UVA (1 mW/cm^2, 18 W, 350 nm, Philips), visible Light (0.44 mW/cm^2, 18 W, fluorescence lamp, Philips) and dark conditions were employed. The distance between the light source and samples were maintained at 30 cm The power used for both UVA and visible light were 18 W. Thus, it could be established that both the light exposures were under similar power densities. The dark condition was maintained by covering the beakers with opaque sheets. The experiments were conducted at 37 °C; 50 rpm in a shaking

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