https://doi.org/10.48048/tis.2022.5696

Toxicity Assessment of Titanium Dioxide Nanoparticles in *E. coli* Mutant with Truncated Lipopolysaccharide (LPS)

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Received: 26 June 2021, Revised: 17 September 2021, Accepted: 24 September 2021

Abstract

As the production and use of titanium dioxide nanoparticles (TiO_2NPs) have increased, so has concern over their environmental impact. Bacteria play an important role in ecological processes and may be harmed by these nanoparticles' toxicity. The specific mechanisms of TiO_2NPs toxicity towards bacteria, as well as the primary role of bacterial cell surface composition, are so far not fully recognized. The impact of TiO_2NPs exposure on both wild strain, *E. coli* K 12 W3110, and LPS biosynthesis pathway *galU* gene mutant was assessed in the present study to gain insight into the underlying mechanisms contributing to TiO_2NPs toxicity. We demonstrate that a truncated LPS causes increased sensitivity to TiO_2NPs. During the experiment, TiO_2NPs formed aggregates with bacteria, and the electrophoretic mobility test revealed a higher interaction between nanoparticles and bacteria due to the positive and negative charge on their surfaces. The high sensitivity of the *galU* mutant to TiO_2NPs may be attributed to the shorter LPS and also possibly with outer membrane efflux proteins like TolC and porins/OmpF, which provide stability to the outer membrane while also acting as permeability barriers to hazardous substances like chemicals, detergents, antibiotics and nanoparticles.

Keywords: Toxicity, Nanoparticles, Titanium oxide, Mutation, LPS

Introduction

Titanium dioxide nanoparticles (TiO_2NPs) are among the most commonly used nanomaterials with a wide range of applications [1-3]. These are used to provide whiteness and opacity to products such as papers, cosmetics, inks, paints, toothpaste, plastics, coatings, and textiles [4]. TiO_2NPs is heavily used in sunscreen as UV protector as it reflects and scatters ultraviolet (UVB and UVA) in sunlight [5]. The widespread usage and high demand for these products undoubtedly affect living organisms and ecosystems. Understanding the toxicity of TiO_2NP in simpler model organisms like bacteria can aid in the risk assessment and safety of these nanomaterials.

Gram-positive bacteria have been reported to be more resistant to nanoparticles toxicity [6-8]. This is because Gram-positive bacteria have a distinct cell wall structure and composition as compared to Gram-negative bacteria. Gram-positive bacteria have cell walls composed of an 80 nm thick peptidoglycan layer containing covalently bound teichoic and teichuronic acids, whereas, Gram-negative bacteria possess a cell wall composed of 1-3 μm thick lipopolysaccharides (LPS) and an 8 nm thick peptidoglycan layer, making it easier for released ions from nanoparticles to enter the cell [9]. As a result of the lack of a strong protective coating of peptidoglycan seen in Gram-positive bacteria, Gram-negative bacteria are more prone to cell wall disintegration induced by physical interaction with nanoparticles. Another possible reason is the lipopolysaccharides (LPS) of Gram-negative bacteria cell walls, which provide a negative charge to their surface that aids in the structural stabilization of the cell wall. Because of the negative charge conferred by LPS, there is greater electrostatic interaction between negatively charged Gram-negative bacteria and positively charged nanoparticles [10]. However, LPS also protects Gram-negative bacteria by generating a permeability barrier that prevents hazardous chemicals like nanoparticles from entering the cell [11]. In Gram-negative bacteria like *E. coli*, LPS production starts with a precursor molecule, UDP-N-acetylglucosamine, which is then acted on by a series of enzymes encoded by the *rfa* genes (also known as *waa* genes) organized in 3 operons to produce lipid A, inner and outer core oligosaccharides, and the O antigen. *galU* gene is essential for the formation of UDP-glucose, an essential precursor for the LPS core oligosaccharide biosynthesis [12] (**Figure 1**).

LPS mutations can distress the stability of the outer membrane, consequentially leading to multiple modifications in cell membrane proteins, penetrability, sensitivity to chemicals like NPs and antibiotics, and also motility [13]. Nevertheless, the precise role of the bacteria's core oligosaccharide of LPS in TiO_2NPs sensitivity or resistance is still not exactly known. To better understand the cellular components involved in TiO_2NPs toxicity at the molecular level, toxicity assessment studies were carried out with *E. coli* MG165 wild strain, *E. coli* K-12 W3110 strain (lacking the O-antigen of LPS) and an insertion mutant of the *galU* gene.

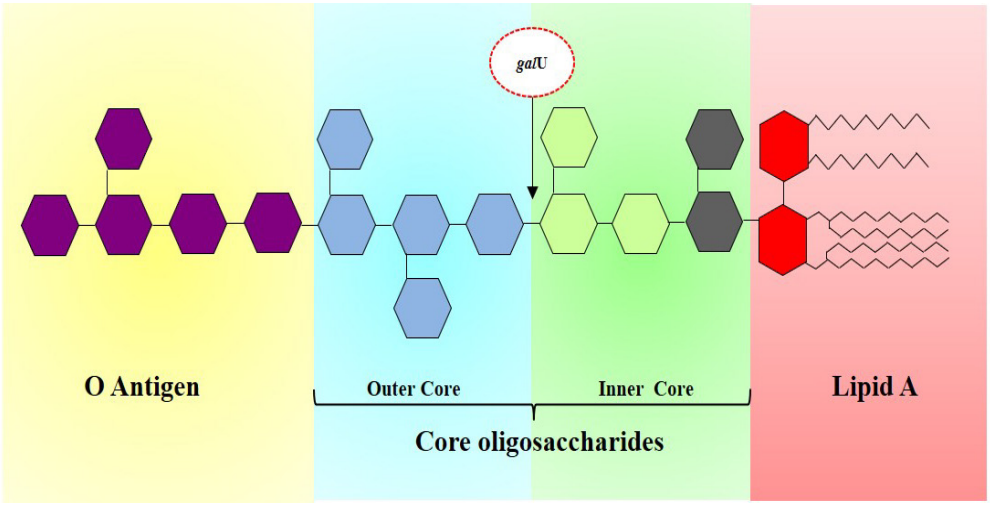


Figure 1 Structure and biosynthesis of LPS in *E. coli* K-12 MG1655. *E. coli* K-12 W3110 possesses rough LPS as it lacks O-antigen repeats. *E. coli* K-12 W3110 insertion *galU* mutant possesses truncated LPS.

Materials and methods

Preparation of TiO_2NPs-P25

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 torutile mixture with a primary particle size of about 21 nm and a specific surface area of 35 - 65 m^2/g. The suspension was prepared by dispersing 100 mg of TiO_2NPs 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 an electron microscopy facility, AIIMS, Delhi. Samples were prepared by evaporating a droplet of nanoparticle suspension on a copper grid at room temperature. The 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 Zetasizer equipment (Nano ZS Malvern Instruments, UK). Measurements were made on colloidal nanoparticle suspensions in milli-Q water.

Bacterial electrophoretic mobility

The electrophoretic mobility of the bacterial cells was measured at 25 °C using Zetaphoremeter (IIT, Delhi, India). An aliquot of freshly harvested bacteria was suspended in a background NaCl solutions (1, 3, 10, 30 and 100 mM) with ~10^7 cells/mL and used for electrophoretic mobility measurement immediately before experimentation. The electrophoretic mobility was measured in triplicate for each ionic strength. Electrophoretic mobilities and zetapotential were recorded for each sample.

Preparation of resting cells

E. coli K-12 MG1655 wild strain, *E. coli* K-12 W3110 and a laboratory generated *E. coli* K-12 W3110 transposon insertion *galU* gene mutant were used for this study. Cells were inoculated in 100 mL of a Luria-Bertani broth (supplemented, when necessary, with 30 μg kanamycin mL^{-1}) and then incubated at 37 °C at 150 rpm until the exponential phase of growth was attained ($OD_{600\ nm}$: 0.5). The cells were next harvested by centrifugation at 8,000×g for 10 min and washed twice with sterile milli-Q water (pH 6). The bacterial suspension obtained was adjusted to an OD_{600} nm of 0.4 (10^9 cells/mL) and used immediately for assays.

Toxicity assessment

Experiments were conducted in 100 mL flasks containing 8 mL of sterile milli-Q water. The 1 mL of the bacterial suspension and 1 mL of the TiO_2NPs stock suspension for 1 gm/L concentration. For lower TiO_2NPs concentration, dilution of TiO_2NPs in milli-Q water was made, and in control (unexposed), only milli-Q water was added. The flasks were then incubated at room temperature on a dark rotary shaker (150 rpm). After 24 h of exposure, the toxicity of TiO_2NPs toward *E. coli* K-12 MG1655 wild strain*, E. coli* K-12 W3110 and *galU* mutant was investigated using the growth curve inhibition, BacLight bacterial viability kit and dehydrogenase activity assay and CFU assay.

Toxicity assessment by growth curve inhibition

To study the toxicity of TiO_2NPs toward bacterial strains, 500 μL of TiO_2NPs 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 OD_{600} nm was measured every hour to monitor the bacterial growth till 20 h. By comparing the absorbances of TiO_2NP exposed and undamaged cells, the percentage of viability was calculated.

Toxicity assessment by dehydrogenase activity assay

Toxicity assessment was done using a rapid and accurate dehydrogenase assay. The reaction mixture was prepared using M9 medium, TiO_2NPs exposed cells, 10 mM NaCl, and 0.05 % 2, 3, 5-Triphenyl tetrazolium chloride (TTC) reagent. Microtiter plates were then incubated at 30 °C with shaking (200 rpm). The amount of TTC conversion by TiO_2NPs exposed cells and unexposed cells were measured using a Microtiter Plate Reader at 480 nm every hour till 20 h.

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.

Toxicity assessment by CFU

CFU was taken by the drop count method, twelve drops (20 μL per drop) were used for each dilution, transferred onto solid Luria Bertani (LB) agar medium, and incubated at 37 °C for 24 h. The toxicity was assessed by comparing the number of colony-forming units (CFU) produced by the exposed and unexposed (control) samples.

Results and discussion

Size characterization of the NP-TiO_2 stock suspension

DLS measurements revealed that the average hydrodynamic diameter of the dispersion decreased significantly, and became relatively constant after 30 min. These findings suggest that the TiO_2NPs stock suspension is largely composed of small aggregates. The zeta potential of TiO_2NP in milli-Q water was 25 mv. TiO_2NPs were positively charged in milli-Q water. TiO_2NPs were found to make aggregates in milli-Q water as shown in **Figure 2**. The hydrodynamic diameter obtained (80 - 1,000 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).

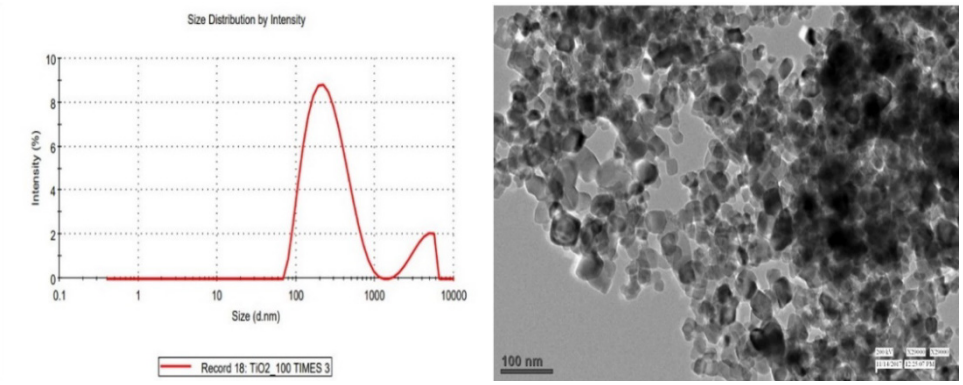
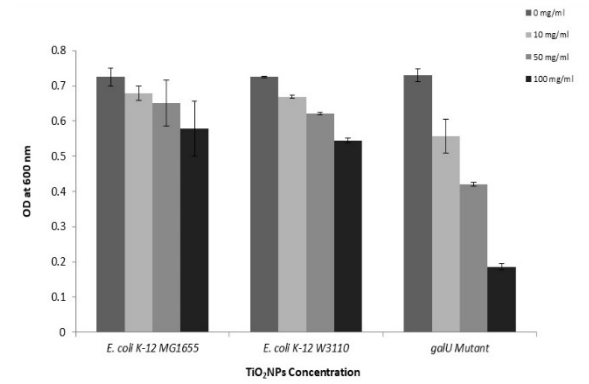


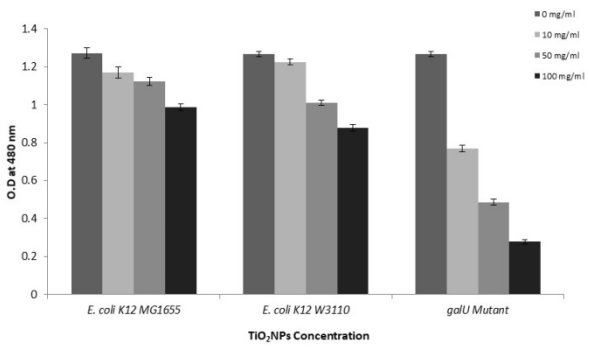
Figure 2 Physical characterization of the TiO_2NPs stock suspension. Particle size distribution by DLS measurement and transmission electron microscopy image of the TiO_2NPs after 20 min of sonication in milli-Q water.

Toxicity assessment

The toxicological effects of TiO_2NPs on *E. coli* K-12 MG1655, *E. coli* K-12 W3110 and *galU* mutant were studied using growth curve inhibition, dehydrogenase activity test and colony forming unit methods. TiO_2NPs showed toxicity towards the wild type strains and *galU* mutant used in this study, but remarkable toxicity was observed in the case of the *galU* mutant that showed 75 % toxicity for TiO_2NPs nanoparticles, respectively, using the growth inhibition method. The dehydrogenase activity assay used to assess toxicity showed 78 % toxicity for TiO_2NPs on the *galU* mutant (**Figures 3(a)** and **(b)**).



(a)



(b)

Figure 3 (a) Cell viability after 20 h of exposure to different concentrations of TiO_2NPs assessed by growth curve inhibition, (b) dehydrogenase activity assay.

Readings obtained by the Live/Dead BacLight bacterial viability assay taken after 0 and 20 h for TiO_2NPs exposed and unexposed samples also showed similar viability results. CFU assay studies were also conducted for TiO_2NPs toxicity, which also demonstrated the highest level of toxicity of TiO_2NPs on *galU* mutant (20 % viable cells), which was in accordance with the results of the growth curve inhibition and dehydrogenase activity assay. The toxicity results suggest a change in the cell permeability due to LPS truncation in the *galU* mutant, leading to increased TiO_2NPs toxicity. Higher levels of sensitivity of the truncated LPS mutant can be attributed to the difference in the thickness of LPS. The *galU* mutant produces short LPS as it carries only the inner core and lacks the outer core oligosaccharides and O-antigen, whereas *E. coli* K-12 strains; MG1655 possess complete LPS and *E. coli* K-12 W3110 lack only the O-antigen of LPS. Another mechanism responsible for the increased TiO_2NPs toxicity in the *galU* mutant could be the outer membrane TolC efflux proteins, which play a key role in the expulsion of various chemicals from the cell, including detergents, toxins, and antibacterial drugs [14]. The *galU* mutation has been found to impair the secretion of TolC protein [15], a major protein of the AcrAB-TolC efflux pump, which has pleiotropic effects including reduced expression of membrane proteins like porin OmpF/C involved in membrane stability, solute transport and signalling as well as increased sensitivity to chemicals, detergents and antibiotics [16]. A combination of these processes might be responsible for the increased toxicity of TiO_2NPs towards the *galU* mutant.

Role of electrostatic interaction in toxicity

The increased toxicity of TiO_2NPs nanoparticles towards the *galU* mutant was initially thought to be due to electrostatic interactions, which is expected to play an essential role in determining nanoparticles toxicity towards bacteria. It has also been reported that the outer membranes of Gram-negative bacteria contain membrane-bound proteins and LPS that can ionize and give rise to a pH-dependent net surface charge [17]. **Table 1** shows the electrophoretic mobility of TiO_2NPs and *E. coli* K-12 MG1655, *E. coli* K-12 W3110, and *galU* mutant in 10 mM NaCl. All bacterial strains were found to be negatively charged and their electrophoretic mobility was found to be negative across the different ranges of the electrolyte concentration. These findings are in line with prior research that found that most bacterial cell wall surfaces are negatively charged at ambient pH due to the presence of many functional groups [18]. In 10 mM NaCl, TiO_2NPs exhibited a net positive charge. Furthermore, as expected, as the concentration of salt (NaCl) increased, the absolute magnitude of the cell zeta potential and electrophoretic mobility dropped [19]. But, despite the reduction in the number of charges within the LPS layer due to truncation, as well as the associated decrease in the volume of the surface layer where the remaining charges are distributed, no significant differences in cell zeta potential and electrophoretic mobility were observed among these strains, including the most sensitive *galU* mutant, which showed the highest-level toxicity for TiO_2NPs.

Table 1 Electrophoretic mobility of TiO_2NPs and *E. coli* K-12 W3110 strains in 10 mM NaCl.

Bacterial strain	Mobility in 10 mM NaCl solution (µm/s/V/cm)
E. coli K-12 MG1655	–2.75
E. coli K-12 W3110	–2.88
galU mutant	–3.67
TiO_2NPs-P25	+2.46

Higher TiO_2NPs toxicity towards *galU* mutant can be due to shorter truncated LPS as compared to wild strains, allowing for increased TiO_2NPs proximity and thus more damage. However, further characterization of LPS will be required to fully identify the role of LPS truncation in the destabilization of a membrane of different strains. The present study indicates that the surface properties of bacteria play a crucial role in TiO_2NP toxicity.

Conclusions

In the present study, *E. coli* K-12 W3110 and an insertion mutant producing truncated LPS were used to better understand the morphological features of bacteria involved in TiO_2NPs toxicity at the molecular level. LPS is the most prevalent component of the outer membranes of Gram-negative bacteria and helps in structural stabilization and protection by creating a permeability barrier that prevents harmful chemicals like nanoparticles from entering the bacterial cell. The *galU* mutant with truncated LPS were found to be more sensitive to TiO_2NPs, indicating that the outer membrane is no longer capable of providing an efficient barrier function. Another proposed cause of increased TiO_2NPs toxicity towards *galU* mutant is changed expression of TolC efflux proteins and OmpF/C porin proteins due to *galU* mutation, which contributes to the instability of the outer membrane and disturbed cell signaling. To identify and understand the molecular determinants responsible for TiO_2NPs sensitivity or resistance, further in-depth research based on proteomics of these outer membrane proteins is required.

Acknowledgements

This work was supported by DBT STAR College Grant and Gargi College, University of Delhi, India.

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