

Titanium Dioxide Nanoparticles Interaction, Uptake and Genotoxicity in *Salmonella typhimurium* Bacteria

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ABSTRACT

Titanium dioxide (TiO₂) nanoparticles (NPs) were characterized using dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), zeta potential analysis and transmission electron microscopy (TEM) with corresponding energy dispersive X-ray spectroscopy (EDS). Genotoxicity experiments with *Salmonella* strains TA98 and TA100 were negative when performed using a miniaturized pre-incubation assay. Flow cytometry experiments indicated a time and dose-dependent interaction of the NPs with the bacteria. Subsequent TEM analysis revealed that although the NPs heavily associated with the bacterial cell wall, only trace amounts could be found within the bacterium. This result suggested limited to no uptake of the NPs by the bacteria.

The lack of uptake of NPs was consistent with the negative results found in the bacterial mutagenicity assays. The results suggest that alternative assays, e.g. mammalian cell-based assays, may be more appropriate for the assessment of the genotoxicity of nanomaterials, in particular TiO₂ NPs.

Keywords: Titanium dioxide nanoparticles, Ames assay, *Salmonella*, bacteria, mutagenicity

1 INTRODUCTION

The Ames bacterial reverse mutation assay is the most widely used test to assess genotoxicity. In recent years the utility of the test for NPs has been called into question due to the apparent inability of bacteria to uptake NPs, a necessary step in assessing genotoxicity. The goal of this work was to determine the ability of several of the Ames assay strains of *Salmonella typhimurium* to interact with and uptake TiO₂ NPs.

2 METHODS

2.1 Characterization of TiO₂ NPs

TiO₂ NPs were provided as a gift from DeGussa AG (Hanau-Wolfgang) at a reported concentration of 560 mg/mL and a reported anatase to rutile phase ratio of 8:2 [1]. The TiO₂ NPs were analyzed using DLS (Malvern Zetasizer Nano ZS), zeta potential analysis (Malvern Zetasizer Nano ZS), NTA (NanoSight NS500) and TEM (JEOL JEM-1400).

2.2 Genotoxicity Experiments

Before addition to the bacteria TiO₂ NPs were diluted in either minimal essential media (MEM, Life Technologies) plus 10% fetal bovine serum (FBS, ATCC) or double distilled water. Bacterial treatment was completed according to a miniaturization protocol which included pre-incubation of samples in microtiter wells followed by plating on minimal Medium E agar plates [2]. One hundred μ L of bacteria (*Salmonella*) were incubated with 10 μ L of vehicle controls, TiO₂ NPs (ranging in concentration), mutagen or TiO₂ NPs plus mutagen for 60 minutes at 37°C with shaking. Mutagens used in this assay were ethyl methanesulfonate (EMS), used for TA100 and 2-nitrofluoranthene (2-NF), used for TA98. Samples were tested in duplicate. After incubation, the contents of each well were plated on minimal Medium E plates [3]. After a 48 hour incubation, revertant colonies were counted using a Symbiosis (Frederick, MD) Protocol 3 colony counter.

2.3 Flow Cytometry Experiments

Salmonella samples (100 μ L) were incubated with 10 μ L of TiO₂ NPs (ranging in concentration) for 60 minutes. Cells were then washed by centrifugation (1,500G, 15 minutes) to remove unattached nanoparticles. After centrifugation the supernatant was removed and cells were resuspended in 5 mL of fresh phosphate buffered solution (PBS). Five μ L of this cell suspension was added to 500 μ L of staining buffer, consisting of PBS with 1% (v/v) Tween 20, along with 5 μ L of propidium iodide (PI).

The stained cell samples were assessed by flow cytometry using a FACS Canto II (BD Biosciences). To prevent the visualization of unbound nanoparticles, non-cellular debris and other noise, only signals that were positive for PI staining were analyzed. Ten thousand (10,000) PI positive events were analyzed per sample.

2.4 TEM Analysis

Salmonella samples (100 μ L) were incubated with 10 μ L of TiO₂ NPs (5.6 μ g/mL in water) in either water or media (MEM + 10% FBS) for 60 minutes. After the 60 minute incubation, samples of bacteria were removed from the microtiter wells and pelleted (20,000G, 30 minutes). The cell pellet was fixed, dehydrated, embedded in resin, and sectioned. The sections (80 nm) were transferred to support grids (Ted Pella, carbon type B, 300 mesh, copper) and imaged using a JEOL JEM-1400 transmission electron microscope at an operating voltage of 80 kV. Scanning transmission electron micrographs were obtained and corresponding EDS analysis was completed on the specimens.

3. RESULTS

3.1 Characterization of TiO₂ NPs

The average particle size determined by DLS and NTA was approximately 130 nm. TEM analysis of the particles indicated a broad size distribution ranging from approximately 20 to 150 nm (Figure 1). TEM analysis also indicated the presence of agglomeration and slight aggregation. The zeta potential of the particles was determined to be approximately -40 mV.

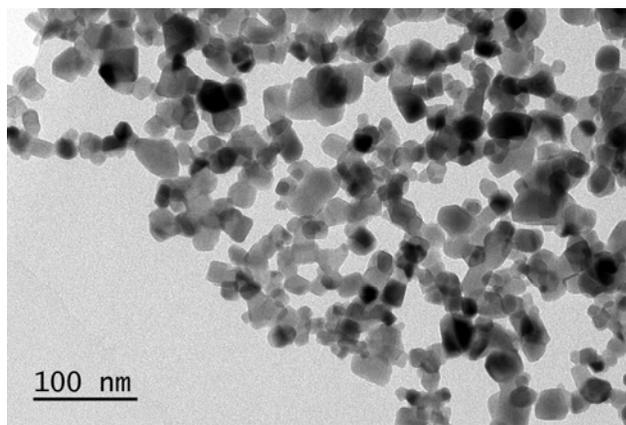


Figure 1: TEM image of TiO₂ NPs as received.

3.2 Genotoxicity Experiments

TiO₂ NPs, at various concentrations, were tested with Salmonella TA98 and TA100 bacteria to determine their mutagenicity. Even with pre-incubation of the TiO₂ NPs with the bacteria, results were negative across a wide range of concentrations of TiO₂ NPs. The results indicated that the particles were not genotoxic.

3.3 Flow Cytometry Experiments

Association between the bacteria and the TiO₂ NPs was time dependant and reached a maximum by 60 minutes. Incubation of the bacteria in water or media (MEM + 10% FBS) did not affect the time course of association between the bacteria and the NPs. Flow cytometry suggested that the particles were interacting with the bacteria.

3.4 TEM Analysis

Transmission electron microscopy was completed in order to better characterize the association between the TiO₂ NPs and the bacteria. A representative scanning transmission electron micrograph of the bacteria incubated with TiO₂ NPs and media (MEM + 10% FBS) is shown in Figure 2 (left hand side). EDS analysis was completed to verify the presence of TiO₂ NPs (Figure 2, right hand side). As Figure 2 shows, there is a substantial amount of TiO₂ NPs surrounding the bacteria yet almost no NPs can be observed within the bacteria. The results suggest that although there is substantial interaction of the NPs with the cell wall of bacterium there is relatively no uptake of the particles into the cell. If the the particles must interact with the DNA or other internal cell components to exert their genotoxic affect such bacterial based genotoxic assays, e.g. Ames assay, might not be appropriate due to the inability of the bacteria to uptake the NPs.

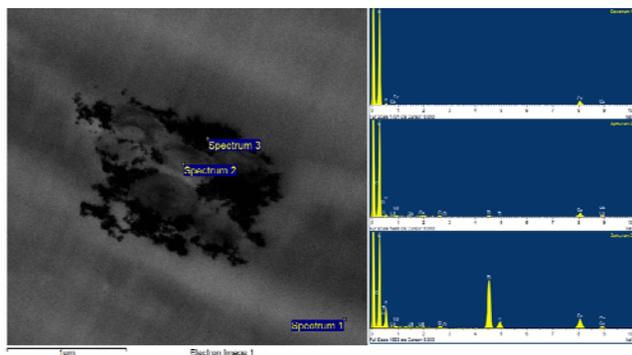


Figure 1: Scanning transmission electron micrograph of TiO₂ NPs interacting with bacteria (in media). The EDS spectrum of each point showed in the figure can be found on the right hand side of the image.

REFERENCES

- [1] A. Patri, T. Umbreit, J. Zheng, K. Nagashima, P. Goering, S. Francke-Carroll, E. Gordon, J. Weaver, T. Miller, N. Sadrieh, S. McNeil and M. Stratmeyer, "Energy dispersive X-ray analysis of titanium dioxide nanoparticle distribution after intravenous and subcutaneous injection in mice," *Journal of Applied Toxicology*, 29, 662-672, 2009.
- [2] R. Elespuru, A. Rajani and K. Butler, "Miniaturized Bacterial Mutagenicity Assays Designed to Use Less Test Agent," *Environmental and Molecular Mutagenesis*, 53, S45-S45, 2012.
- [3] D.M. Maron and B.N. Ames, "Revised Methods for the Salmonella Mutagenicity Test," *Mutation Research*, 113, 173-215, 1983.