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THE INFLUENCE OF UV LIGHT ON THE GENOTOXICITY OF ENGINEERED NANOPARTICLES

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Background

The increasing amounts of engineered nanoparticles (ENPs) in the market and their novel properties call for rapid screening of their toxicological properties. Some of the most commonly studied toxicological endpoints to ENP exposure involve cytotoxicity, effects on growth and reproduction, and mortality [1]. However, in the recent years there has been growing evidence that various ENPs can induce genotoxicity [1, 2]. Previously, presence of ROS has been insinuated to play a role in genotoxic effects of particles, which may derive from various factors including surface type, shape and crystallinity [3]. Exposure to UV light is able to create radicals and reactive oxygen species (ROS) in particles with photocatalytic activity, such as TiO_2 ENPs. Due to this property and the widespread of use, including proucts such as sunscreens and cosmetics the aims of this study were to:

1) investigate the genotoxic and photogenotoxic effects of 3 different TiO_2 ENPs
2) evaluate the feasibility of a high-throughput *umu* test in detecting photogenotoxicity
3) observe differences in response between three TiO_2 NPs differing in crystalline structure and surface modification.

Initially, the maximum ROS production from TiO_2 ENPs in response to UV light was measured in the presence of methylene blue dye. The effect of UV light on bacterial viability and growth was also measured over time.

References
[1]. Wang et. al. 2013. Engineered nanoparticles may induce genotoxicity. *Environ. Sci. Tech.* 47 (23), 13212-13214.
[2]. Magdolenova et. al. 2014. Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered nanoparticles. *Nanotoxicology* 8 (3), 233-278
[3]. Schins 2002. Mechanisms of genotoxicity of particles and fibers. *Inhal. Toxicol.* 14 (1), 57-78.

Materials and Methods

Nanoparticles and stock suspension preparation

TiO_2 NM-105 (anatase-rutile, 84-16%, uncoated), TiO_2 NM-104 (rutile, surface modification hydrophilic), and TiO_2 NM-103 (rutile, surface modification hydrophobic) employed in this study were received from the depository of the European Commission Joint Research Centre (JRC) (Ispra, Italy). Stock suspensions of TiO_2 ENPs were prepared at concentration of 0.1 and 1 mg/mL in MilliQ H_2O and sonicated for 20 minutes at 10% Amplitude using a Branson Digital Sonifier Model 250. TiO_2 NM-103 (hydrophobic) was pre-wetted using 0.1 vol % ethanol and turned into a paste form with the use of a spatula, followed by subsequent addition of MilliQ H_2O and sonication.

TiO_2 ENPs ROS production in the presence of UV light

The UV lamp used was Philips original home solarium, with highest intensity peak at 350 nm and a power of 225 mW/cm² at 35 cm distance from the sample. Methylene blue dye was ordered from Merck (Germany). Degradation of the methylene blue dye in the presence of UV light and TiO_2 NM-104 ENPs was used as a rapid detection method for ROS formation.

***Umu* Test**

The *umu* genotoxicity test measures the ability of chemicals to induce *umu* gene expression in *Salmonella typhimurium* (TA1535/pSK1002). This strain, containing a *umuC-lacZ* fused gene which is part of the SOS pathway, gets induced in response to genotoxic compounds. Gene induction is estimated by β-galactosidase activity of the fusion gene. Moltox UMU water, wastewater, & concentrated & solid sample test kit (#31-400) was purchased from Trinova Biochem (Germany). The Moltox® *umu*-test is conducted in 96-well microplates according to manufacturer's instructions, which is adapted from ISO 13829. Half of Plate A was covered with aluminum foil to serve as control against UV light which was illuminated for the selected amounts of time. This plate was then incubated for 2 hours at 37 ± 1°C. After incubation, Plate A was then transferred to Plate B and absorbance was read at 620 nm using a microplate reader Multiscan FC from Thermo Scientific. Growth of Plate B was measured after 2 hours of incubation, and post/pre-incubation values were used to calculate the growth rate of Plate B (see Equation 1). Colometric change of ortho-Nitrophenyl-β-galactoside (ONPG), was then measured in Plate C at 405 nm. Determination of bacterial cell growth and genotoxicity after exposure to different amounts of UV light was also completed using the reagents from Moltox kit.

Experimental Setup

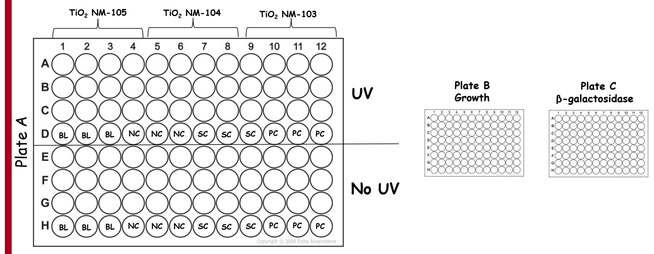


Figure 1: Experimental design using 96-well plate. The plate was prepared in two identical parts, where one half was exposed to UV light and the other half covered with aluminum foil, including negative and positive controls. Serial dilutions were performed for the three TiO_2 ENPs (NM-105, NM-104, NM-103) to achieve four concentrations, respectively. N=3 BL=Blanks, NC=Negative Control, SC=Solvent Control, PC=Positive Control

Engineered Nanoparticle Characterization

TiO_2 ENPs	Concentration	Medium	z-avg (nm)	CV (%)	PDI	CV (%)	z-potential (mV)	CV (%)
NM-105 (24.3 ± 3.5 nm)	1 mg/mL stock	MilliQ	106.8	2.9	0.22	2.3	28.8	4.2
	666.7 µg/mL	TGA	382.6	6.5	0.22	9.5	-8.2	44.6
	0.1 mg/mL stock	MilliQ	106	1.7	0.22	8.5	13.1	28.5
	66.7 µg/mL	TGA	262.7	4.9	0.24	2.9	-16.8	10.8
NM-104 (25.0 ± 1.7 nm)	1 mg/mL stock	MilliQ	98.7	1.37	0.34	1.8	20.3	8.7
	666.7 µg/mL	TGA	94.6	1.5	0.23	2.6	-21.6	4.5
	0.1 mg/mL stock	MilliQ	93.1	0.2	0.24	0.7	22.7	3.2
	66.7 µg/mL	TGA	96.5	1.7	0.22	4.5	-23.4	6.9
NM-103 (24.7 ± 2.3 nm)	1 mg/mL stock	MilliQ	97	2.9	0.36	5.5	20.8	7.7
	666.7 µg/mL	TGA	98.4	1.4	0.33	3.6	-21.7	4.5
	0.1 mg/mL stock	MilliQ	92.1	0.7	0.24	5.5	25.1	18.3
	66.7 µg/mL	TGA	103.2	1.4	0.26	1.9	-22.6	2.2

TiO_2 NM-105 revealed to agglomerate in the presence of TGA medium

Figure 2: ENP characterization by dynamic light scattering (DLS). Two different concentrations of stock suspension (0.1 and 1 mg/mL) were prepared in MilliQ water for each ENP. Respective test suspensions were prepared at the highest concentrations (66.7 and 666.7 µg/mL) in MilliQ water and 10x TGA media as in the test setup. Average zeta size (z-avg), polydispersive index (PDI) and zeta potential were measured using Zeta Sizer Nano (Malvern) in triplicates (N=3) and given as an average value with a percent coefficient of variance (CV).

TiO_2 ROS formation in the presence of UV light

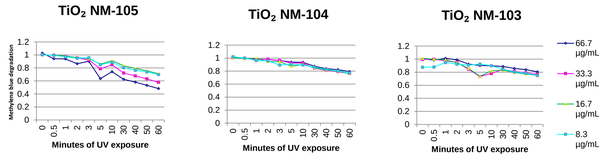
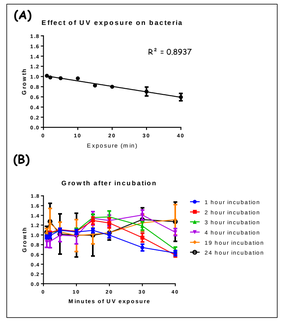
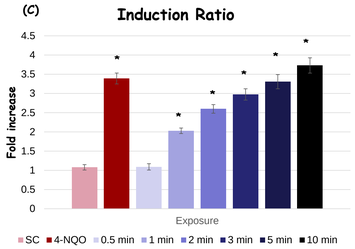


Figure 3: Degradation of 12 µg/mL methylene blue as a scavenger of ROS produced from photocatalytic activity of three TiO_2 ENPs illuminated for 0.5, 1, 2, 3, 5, 10, 30, 40, 50 and 60 minutes. ENP and TGA concentrations were the same as in the test setup in Plate A. N=1 including four concentrations per exposure.

Effect of UV light on bacterial growth and genotoxicity





UV light affects viability/growth of bacteria and causes genotoxicity in exposure times >0.5 min

Figure 4: Effect of UV light on growth (A, B) and genotoxicity (C) of *Salmonella typhimurium* expressed as fold change over control. Graph A represents the decrease in bacterial count of Plate A after exposure to UV up to 40 minutes. Graph B represents growth of Plate A during the 1, 2, 3, 4, 19 and 24 hour incubation period. Graph C represents a separate experiment with lower UV exposure time of 0.5, 1, 2, 3, 5 and 10 minutes where photogenotoxicity was measured using the *umu* test. N=4-8 replica. Data was considered statistical significance when p≤0.05.

Quantification of the Growth Factor (G) and Induction Ratio (IR)

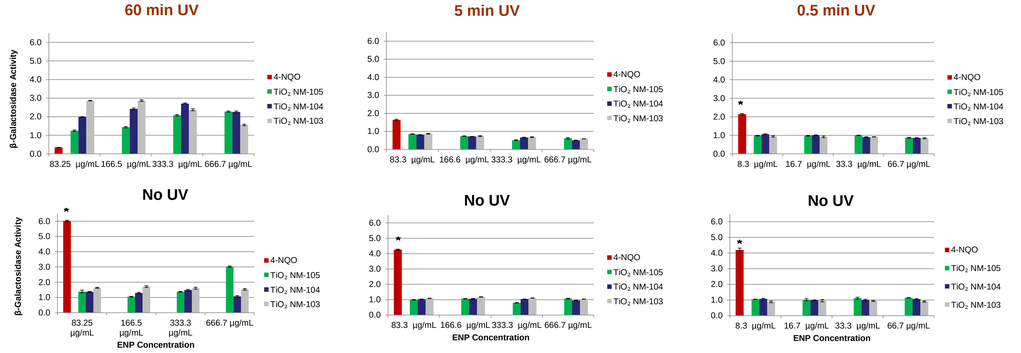
$$G = \frac{A_{620}\,S_{t=2} - A_{620}\,BL \,/\, A_{620}\,NC_{t=2} - A_{620}\,BL}{A_{620}\,S_{t=0} - A_{620}\,BL \,/\, A_{620}\,NC_{t=0} - A_{620}\,BL}$$

$$IR = \frac{1}{G} \times \frac{A_{405}\,S - A_{405}\,BL}{A_{405}NC - A_{405}\,BL}$$

The high background of some ENPs could be interpreted as cell growth using the current ISO standard calculations. Dividing by the pre-incubation values accounts for the shading effect of ENPs, and variation of cell number in the different wells

Equation 1: Calculation of Growth Factor (G) and Induction Ratio (IR). *G* was calculated by dividing the post-incubation with pre-incubation values of Plate B. A_{620} = Absorbance at 620 nm, A_{405} = Absorbance at 405 nm, S = sample, NC = Negative Control, BL = Blank.

TiO_2 ENP Genotoxicity



Damage to the cells?! -Test criteria not met

Protective effect?!

No effect at low ENP concentration+lower UV exposure

Figure 5: β-galactosidase activity of *S. typhimurium* normalized to control for exposure to: high ENP concentration high UV exposure (60 min), high ENP concentration medium UV exposure (5 min), and low ENP concentration low UV (0.5 min).

Summary

ENP characterization

- TiO_2 ENPs agglomerated slightly in MilliQ H_2O, however, NM-105 revealed greater sizes in the presence of 10x TGA medium.

TiO_2 ROS formation in the presence of UV light

- Greatest amount of methylene blue degradation occurred at relatively high amounts of UV exposure time (60 min) and this degradation was greater for the anatase-rutile structured TiO_2 NM-105.

Effect of UV on growth and genotoxicity of bacteria

- Presence of UV light (full spectrum) caused a decrease in bacterial cell count which dropped to 80% after 15 minutes and to 60% after 40 minutes of exposure.
- The decrease in cell count from UV light recovered during the incubation period due to bacterial multiplying and growth.
- Despite this recovery in cell count, UV exposures of higher than 0.5 minutes were genotoxic to bacteria.

TiO_2 ENP Genotoxicity

- Calculation of growth factor in Plate B should include data from pre-incubation (t=0) to account for the background of ENPs and variation in cell number.
- Exposure of *Salmonella typhimurium* to extended periods of UV exposure (60 min) in the presence of TiO_2 ENPs revealed increased β-galactosidase activity relative to controls. However, the UV damage to the bacterial cells makes these results unreliable.
- Shorter UV exposure times (0.5 min) were not sufficient to photocatalytically activate TiO_2 ENPs and no difference was observed between the different sturucture.

Based on these results, the UMU test could potentially be feasible to test photogenotoxicity under the circumstances where UV light is able to cause photoactivation of compounds/nanoparticles without causing damage to the bacterial cells. More recommendations on such settings will be provided in future work.