SIZE AS AN IMPORTANT FACTOR IN NANO-TiO_2 TOXICITY IN MACROPHAGE-LIKE CELLS

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Abstract

Toxicity of TiO_2 nanoparticles (NPs) depends on characteristics of NPs such as nanoparticle shape, size, crystal structure, zeta potential, aggregation and agglomeration tendency, surface characteristics and coatings. However, their effect on toxicity remains unclear due to ambiguous results from different studies. The presented study examined the cytotoxic effect of ten diverse TiO_2 NPs without photoactivation in human monocytic cell lines THP-1 differentiated into macrophage-like cells.

A set of NPs consists of 5 variants of anatase and 5 variants of rutile nanoparticles differing in their diameter (from 3 to 165 nm). TiO_2 samples were characterized in the powder form and dispersed in water and cell culture media. Three cytotoxicity assays were used: MTS, WST-1, and LDH. For all nanomaterials, three independent repetitions were carried out.

Overall, cytotoxicity of all NPs was low even at the highest concentration of 256 µg/ml. The viability of cells did not decrease below 60% for WST-1 and MTS assays and 80% for the LDH assay. Besides concentration, crystalline size was identified as the most important cytotoxic factor. Clear nonlinear relationship between crystalline size and cytotoxicity was detected; higher toxicity induced NPs within the size range 20-60 nm. Increased cytotoxicity in given diameter size range would give an answer to inconsistent findings at size and cytotoxicity relationship.

Keywords: TiO_2, nanoparticles, macrophages, cytotoxicity, nanoparticle size

1. INTRODUCTION

Titanium dioxide (TiO_2) is one of the most widely used material in nanoparticle production. TiO_2 nanoparticles (particles with diameter less than 100 nm; NPs) possess different and more desirable properties as compared to their larger counterparts including enhanced ultraviolet radiation absorption capabilities and photocatalytic or colouring properties. They are commonly used in consumer products such as cosmetics, sunscreens, tooth pastes, and food [1]. However, concerns have been raised that the same properties may increase NPs' bioavailability and could be potentially more harmful for human and other organisms than their bulk forms which are considered to be inert and safe for human health for decades [2-4]. An evidence exists that TiO_2 in nanoform is more toxic than bulk material [5] and other recent studies reported that broad using of TiO_2 nanomaterial may not be safe in consumer products [6] or as photocatalysts [7]. Toxicity of TiO_2 NPs depends on physico-chemical characteristics such as shape, size, crystal structure, zeta potential, aggregation and agglomeration tendency, surface characteristics and coatings [8, 9]. However, their effect on toxicity remains unclear due to ambiguous results from different studies [10].

Numerous *in vivo* studies investigated the distribution of TiO_2 NPs in different organs. They revealed that intravenous administration of TiO_2 NPs in rats mostly led to the accumulation of nanoparticles in liver and spleen, probably due to the high population of macrophages in these organs [11,12]. As cleaners of the body environment, macrophages play an irreplaceable role in nanoparticle removal, immune response, and inflammation development and could be considerably affected by TiO_2 NPs exposure.

The presented study aims to examine the cytotoxic effect of ten diverse TiO2 NPs without photoactivation on human monocytic cell line (THP-1) representing a relevant model of macrophage-like cells.

2. MATERIALS AND METHODS

2.1. Nanoparticles

Different TiO_2 nanoparticles used in the study are listed in **Table 1**. Technical names of NPs based on NPs characteristics were derived as follows:

Anatase/rutile (A/R), size in nm (number), particles (P), no coating/silica coating (-/S)

Table 1 The physical properties of the nano-TiO_2 as provided by the manufacturers

Technical name	Source	Declared size	Declared crystallinity
A5P-	MK Nano	< 5 nm	Anatase
A15P-	US Research Nanomaterials	15 nm	Anatase
A30P-	US Research Nanomaterials	30 nm	Anatase
A50P-	MK Nano	50 nm	Anatase
A100P-	MK Nano	100 nm	Anatase
R30P-	US Research Nanomaterials	30 nm	Rutile
R30PS	US Research Nanomaterials	30 nm	Rutile
R50P-	US Research Nanomaterials	50 nm	Rutile
R100P-	US Research Nanomaterials	100 nm	Rutile
R165P-	US Research Nanomaterials	165 nm	Rutile

2.2. Physicochemical characterization

TiO_2 NPs were characterized in the powder form using following methods: x-ray diffraction (crystallinity), thermogravimetric analysis (mass loss) and Brunauer Emmet Teller measurements (specific surface area).

After dispersion, the size distribution in water and cell culture medium and zeta potential in cell culture medium were measured by dynamic light scattering using Malvern Instruments Zetasizer Nano-ZS instrument.

2.3. Cytotoxicity

To enhance comparability of our results with data produced by other European laboratories as well as to ensure their compatibility with nanotoxicological databases prepared in scope of several ongoing EU projects (e.g. NANoREG, COST Action TD1204 MODENA), standardized protocols for cell cultivation, preparation of NM dispersion and cytotoxicity assays developed within NANOGENOTOX [13] and NANOVALID projects were used. The NANOVALID protocol originally designed for the MTS assay and A549 cells was further modified for using of WST-1 and LDH assays, and THP-1 cell line. Both NANOGENOTOX and NANOVALID are EU FP7 projects whose major aims include development and exchange of best practices in risk assessment and risk management of nanomaterials. Briefly, cells were seeded in 96 well microplates at concentration of 50 000 cells/well (PMA-differentiated THP-1 cells in RPMI supplemented with 10 % heat-inactivated FBS) 24 hours before treatment. Each NP stock suspension in ddH_2O with 0.05% BSA was prepared by probe sonication (400 W and 10 % amplitude, sonicated for 16 minutes in ice bath) [13]. The freshly prepared stock suspension at concentration of 2.56 mg/ml was diluted in cell culture medium to reach the highest tested concentration of 256 µg/ml. A pre-prepared dosing plate containing cell culture medium with ddH_2O and 0.05%

BSA was used to promptly perform serial dilution to obtain 6 concentrations ranging from 16 to 256 µg/ml. The content of the dosing plate was transferred to the appropriate well of the cell plate.

Results of the cytotoxic assays were expressed as percentage of negative control.

3. RESULTS AND DISCUSSION

3.1. Particle characterization

Analyses confirmed anatase structure in all samples declared as being anatase by suppliers. However, one of the rutile sample (30 nm silicon-coated particles, R30PS) was shown to be a mixture of minority rutile and prevailed anatase. The surface area measurements detected lower than expected values for 5 nm anatase particles and uncoated anatase 50 nm particles (A50P-) indicating that sizes of these particles are actually larger. In contrast, A100P- exhibited larger surface corresponding to smaller particles. Crystallite sizes were mainly in accordance with particle diameter calculated for spheres from specific surface area (**Figure 1**), however R30P-, R50P- and R100P- showed smaller crystallite size than expected from specific surface area indicating an aggregation/agglomeration issue. Mass losses as measured by thermogravimetric analysis were generally low (up to 5%) except for A5P- which was shown to contain chloride compound (probably derivative of ethylchloride as derived from thermogravimetric curves).

Table 1 The physical properties of the NMs

	Crystal structure	Crystallite size [nm]	Specific surface area [m^2/g]	Mass loss [%]
R30P-	rutile	28.3	26.9	0.97
R30PS	anatase	42.4	41.8	1.34
R50P-	rutile	27.9	27.0	0.95
R100P-	rutile	54.4	17.6	0.44
R165P-	rutile	145.1	9.0	0.61
A5P-	anatase	10.1	135.8	13.74
A15P-	anatase	27.2	87.3	4.23
A30P-	anatase	28.3	50.4	2.01
A50P-	anatase	20.6	78.4	3.04
A100P-	anatase	88.3	19.8	0.77

Majority of the NPs had the biggest hydrodynamic diameter (Z-Avg) at the highest concentration (batch), and Z-Avg decreased along with decreasing NPs concentrations in the medium. However, the smallest particles (A5P-) behaved differently; in the concentration of 256 µg/ml in the medium they had bigger Z-Avg then theirs batch dispersions. After 24h Z-Avgs remained stable or slightly risen except for A30P- which seemed to decrease in diameter. However, this decrease is most probably caused by the fact that bigger agglomerates settled during the sample stabilisation time and are only detected only in the very beginning of the measurement or are not detected at all by the instrument.

Zeta potential NMs in the medium varied between -13.8 and -22 mV and did not change after 24h.

3.2. Cytotoxicity of TiO_2 NPs

Overall, cytotoxicity of all NPs was low even at the highest concentration of 256 µg/ml. The viability did not decrease below 60% for WTS-1 and MST assays and 80% for the LDH assay (**Figure 1**).

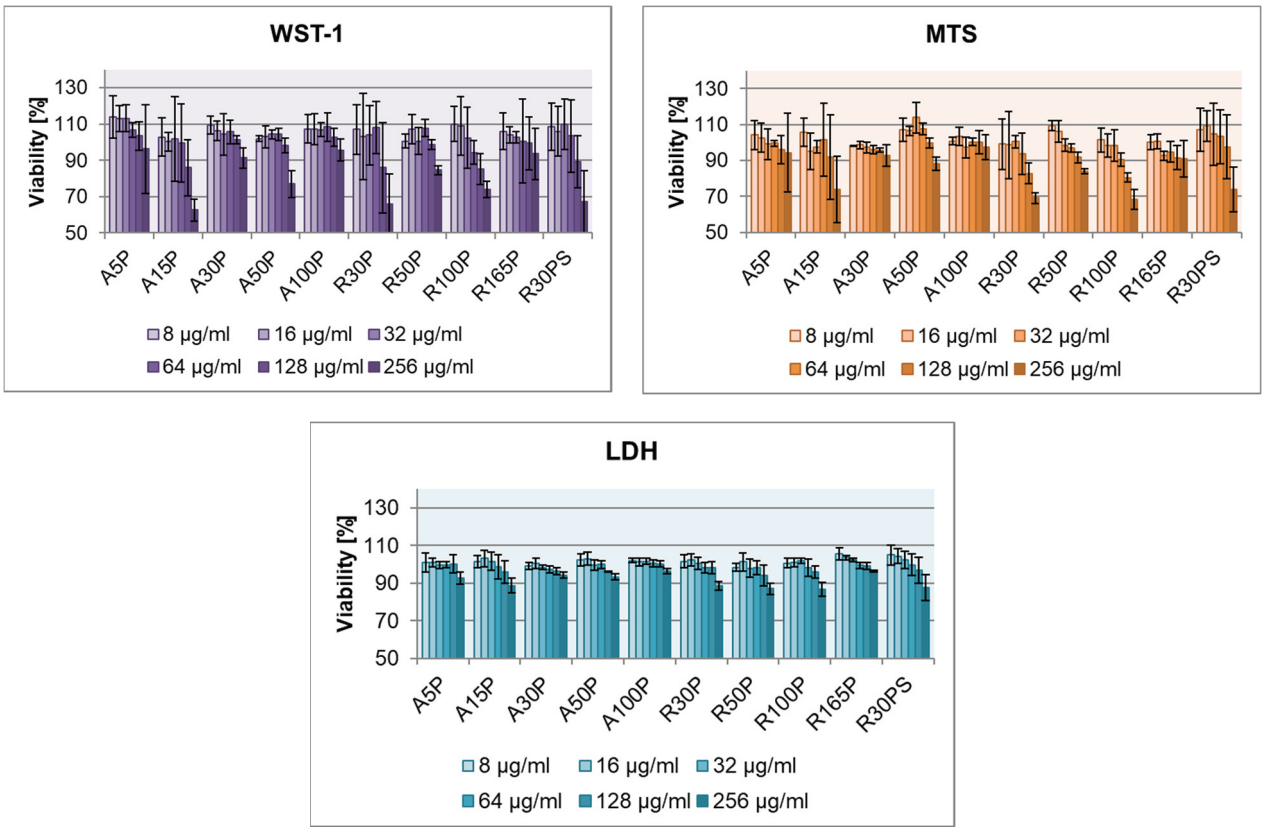

Figure 1 Cytotoxicity induced by TiO2 NMs using three different viability tests (WST-1, MTS, LDH)

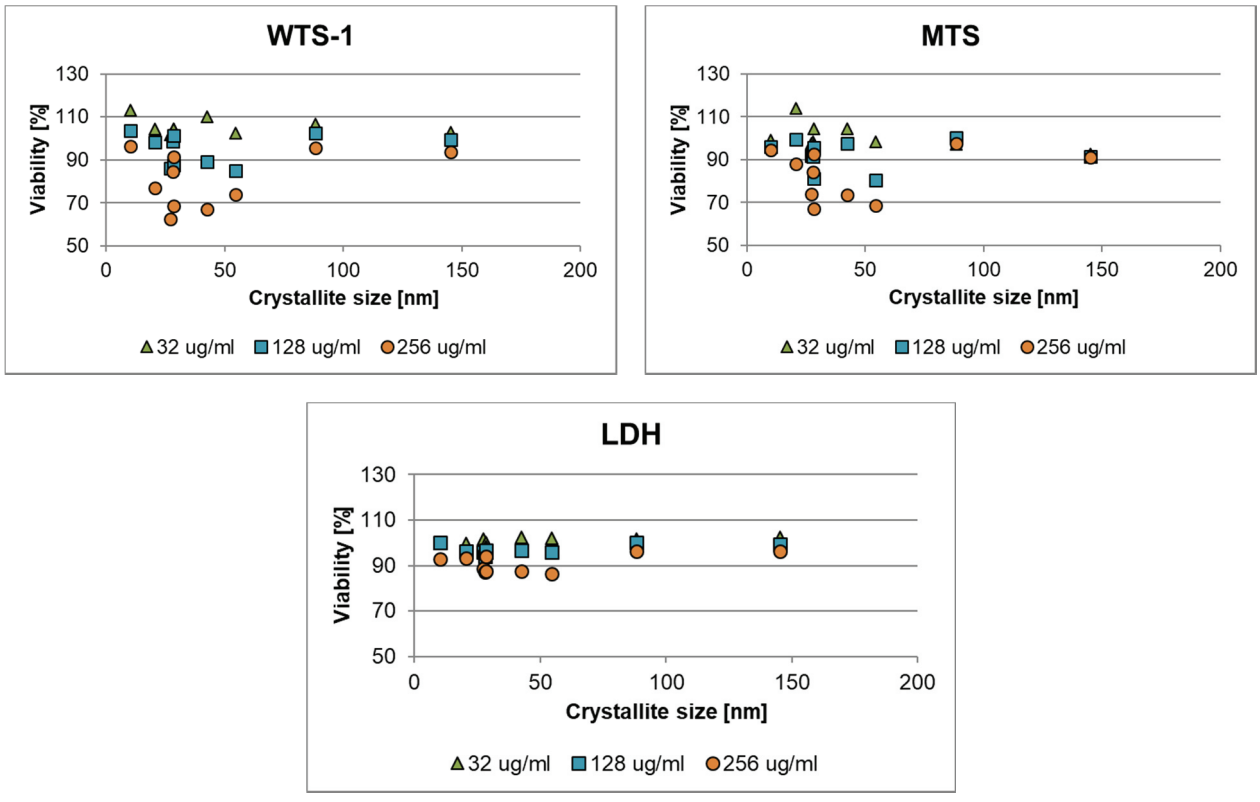

Figure 2 Relation between crystallite size and viability of different assays

Due to inconsistent results between studies dealing with size of nanoparticles and cytotoxic effects, we focused on the relation between crystallite size of particles and cytotoxicity. **Figure 2** suggests a nonlinear relation; the smallest particles had a low cytotoxic effect, then cytotoxicity increased for particles with diameter between 20 - 60nm, and again there was low cytotoxicity for larger particles.

Then we built stepwise linear regression models for all cytotoxicity assays to identify physical properties that possibly affected cytotoxicity. The only stable significant variable for all linear regression models was concentration:

WST-1 ~ - 0.099 Conc.ug.ml** - 32.42 Crystal (rutile/anatase) *

MTS ~ - 0.072 Conc.ug.ml **

LDH ~ - 0.037 Conc.ug.ml ***

** p-value <0.05, ** p-value <0.01, *** p-value <0.001*

4. CONCLUSION

We evaluated cytotoxic effect of 10 diverse TiO_2 NPs in model macrophage cell line representing cells playing a key role in potential interaction, internalization and clearance of TiO_2 NPs. Using three different assays we thoroughly evaluated the effect of nanoparticles on cell viability. Our results demonstrated a nonlinear relationship between toxicity of TiO_2 NPs and the crystallite size. We also observed that particles of a size between 20 - 60 nm were more toxic while smaller and larger ones exhibited low cytotoxicity. We did not find significant difference between anatase/rutile crystallinity of NPs.

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REFERENCES

[1] WEIR, A., WESTERHOFF, P., FABRICIUS, L., HRISTOVSKI, K., VON GOETZ, N., Titanium Dioxide Nanoparticles in Food and Personal Care Products, *Environmental Science & Technology*, 2012, vol. 46, no. 4, pp. 2242-2250.

[2] OPHUS, E.M., RODE, L., GYLSETH, B., NICHOLSON, D.G., SAEED, K., Analysis of titanium pigments in human lung tissue, *Scandinavian Journal of Work, Environment & Health*, 1979, vol. 5, no. 3, pp. 290-296.

[3] HEXT, P.M., TOMENSON, J.A., THOMPSON, P., Titanium dioxide: inhalation toxicology and epidemiology., *Annals of Occupational Hygiene*, 2005, vol. 49, no. 6, pp 461-72.

[4] CHEN, J.L., FAYERWEATHER, W.E., Epidemiologic study of workers exposed to titanium dioxide, *Journal of Occupational Medicine*, 1988 , vol. 30, no. 12, pp. 937-942.

[5] GUICHARD, Y., SCHMIT, J., DARNE, C., GATÉ, L., GOUTET, M., ROUSSET, D., RASTOIX, O., WROBEL, R., WITSCHGER, O., MARTIN, A., FIERRO, V., BINET, S., Cytotoxicity and genotoxicity of nanosized and microsized titanium dioxide and iron oxide particles in Syrianhamster embryo cells., *Annals of Occupational Hygiene*, 2012, vol. 56, no. 5, pp. 631-644.

[6] GUO, Z., MARTUCCI, N.J., MORENO-OLIVAS, F., TAKO, E., MAHLER, G.J., Titanium dioxide nanoparticle ingestion alters nutrient absorption in an in vitro model of the small intestine, *NanoImpact*, 2017, vol. 5, pp. 70-82.

[7] KEBEDE, M.A., VARNER, M.E., SCHARKO, N.K., GERBER, R.B., RAFF, J.D., Photooxidation of ammonia on TiO2 as a source of NO and NO_2 under atmospheric conditions., *Journal of the American Chemical Society*, 2013, vol. 135, no. 23, pp. 8606-8615.

[8] SHA, B., GAO, W., CUI, X., WANG, L., XU, F., The potential health challenges of TiO_2 nanomaterials., *Journal of Applied Toxicology*, 2015, vol. 35, no. 10, pp.1086-1101.

[9] ZHANG, X.C., LI, W., YANG, Z., Toxicology of nanosized titanium dioxide: an update., *Archives of Toxicology*, 2015, vol. 89, no 12, pp. 2207-2217.

[10] SHI, H., MAGAYE, R., CASTRANOVA, V., ZHAO, J., Titanium dioxide nanoparticles: a review of current toxicological data., *Particle and Fibre Toxicology*, 2013, vol. 10, no. 15.

[11] XIE, G., WANG, C., SUN, J., ZHONG, G., Tissue distribution and excretion of intravenously administered titanium dioxide nanoparticles., *Toxicol Letters*, 2011, vol. 205, no. 1, pp. 55-61.

[12] SHINOHARA, N., DANNO, N., ICHINOSE, T., SASAKI, T., FUKUI, H., HONDA, K., GAMO, M. Tissue distribution and clearance of intravenously administered titanium dioxide (TiO_2) nanoparticles., Nanotoxicology, 2014, vol. 8, no. 2, pp. 132-141.

[13] JENSEN, K.A., KEMBOUCHE, Y., CHRISTIANSEN, E., JACOBSEN, N.R., WALLIN, H., GUIOT, C., SPALLA, O., WITSCHGER, O. Final Protocol for producing suitable manufactured nanomaterial exposure media." NANOGENOTOX deliverable report n°3, 2011, 34 pp. Availible online: https://www.anses.fr/en/system/files/nanogenotox_deliverable_5.pdf (Accessed 6th September 2016).