Methodological approaches for potential *in vitro* toxicity assessment of manufactured nanomaterials

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Istituto Superiore di Sanità

NanoInnovation Conference,
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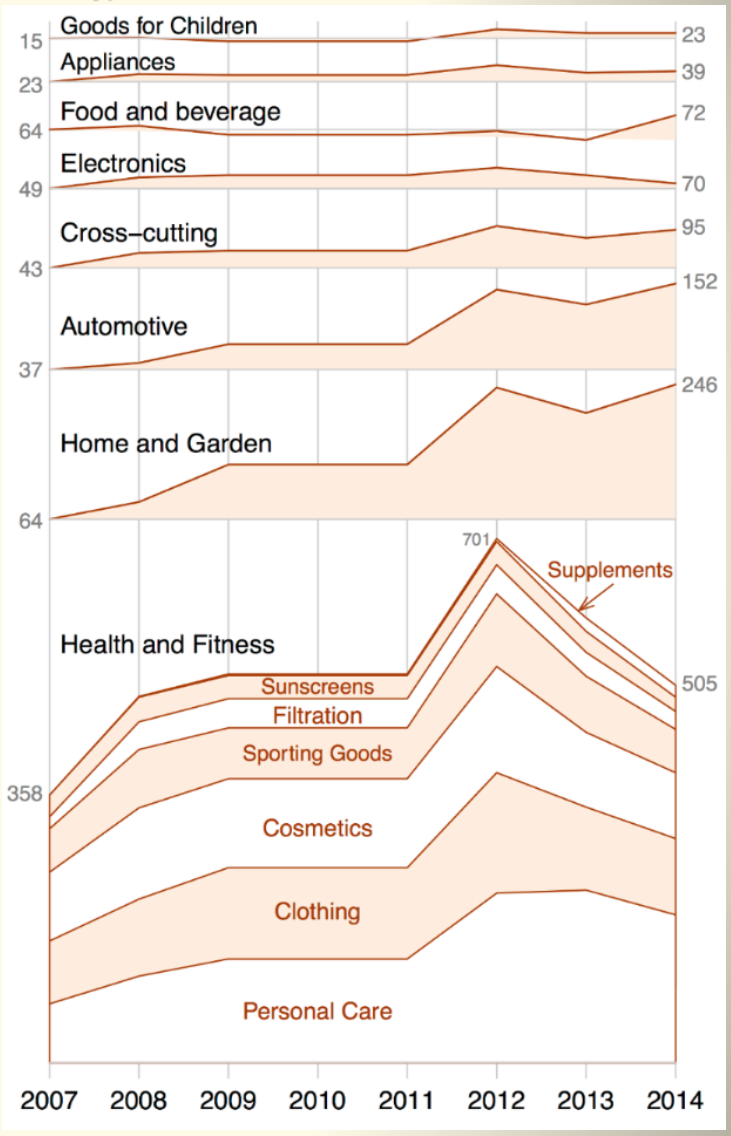
Nanomaterials in consumer products

(a possible challenge for safety)

Due to their physico-chemical properties nanomaterials have large application in consumer products. Currently over 1200 products containing NMs are on market

Nanotechnology Consumer Products Inventory
http://www.nanotechproject.org/cpi/

However possible adverse interaction with biological systems unpredictable on chemical composition basis may occur.



Vance, M. E. et al. (2015) *Beilstein Journal of Nanotechnology*, 6, 1769-1780. http://dx.doi.org/10.3762/bjnano.6.181 Prev Next

Are the assays applied for the safety assessment of traditional chemicals also suitable for the safety assessment of nanomaterials?

Recommendation of the Council on the Safety Testing and Assessment of Manufactured Nanomaterials

19 September 2013 - C(2013)107

The Recommendation notes the importance of the OECD Test Guidelines for the Safety Testing of Chemicals, concluding that many of the existing guidelines are also suitable for the safety assessment of Nanomaterials

At the same time, it recognizes that some guidelines may need to be adapted to take into account the specific properties of Nanomaterials

ISS involvement in NM safety assessment

So far no uniform and standardized methods for NMs suspension/characterization have been used

In Nanoreg an EC FP7 project common protocols for NM suspension and characterization have been applied

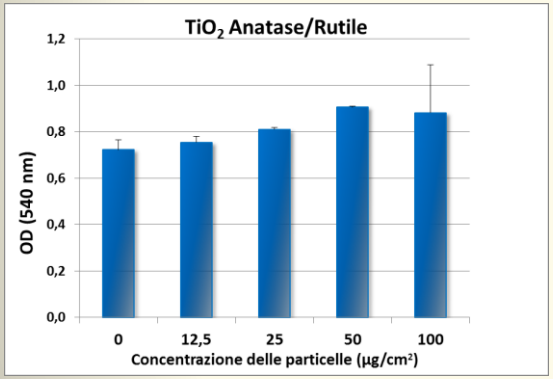
NANOREG
A common European approach to the regulatory testing of nanomaterials

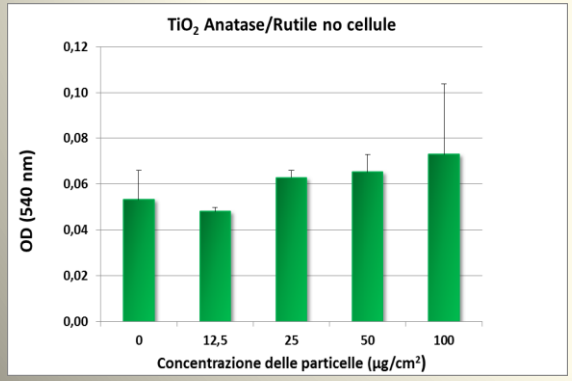
All partners suspended NMs in a Water/BSA solution and sonicated samples for a fixed time in order to fit the previously established parameters based on calorimetric methods

In order to compare results among partners a standard procedure to measure Z-average and Polydispersity index by DLS of batch dispersions was applied. DLS analysis has been performed also after dilution in culture media at the beginning and the end of cell treatment

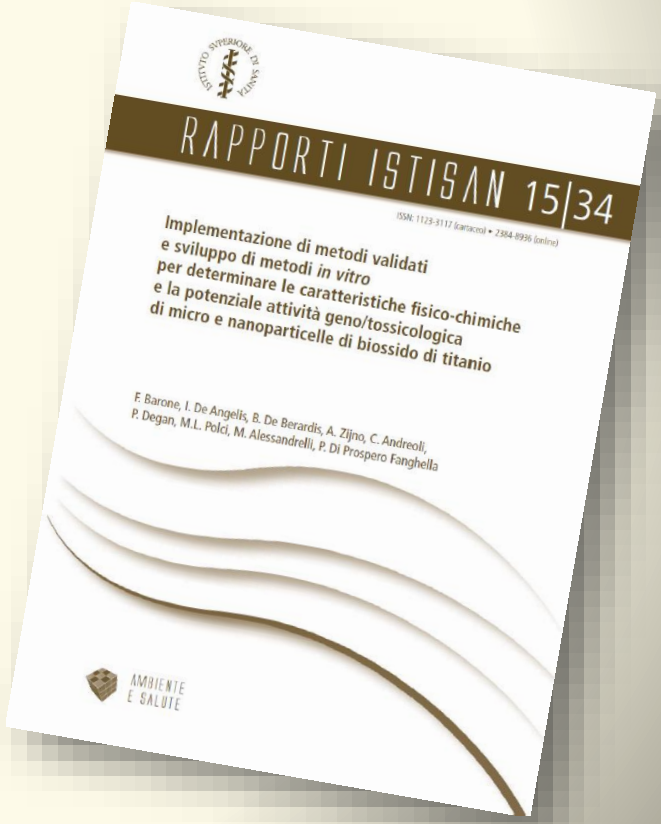
Critical aspects : cytotoxicity assays

Different NMs can interfere with the assays commonly used to determine the toxic effect on cell cultures. Due to their large surface area, NMs can bind to reagents used in the assay, interfering with absorbance or fluorescence in the colorimetric/fluorimetric assays



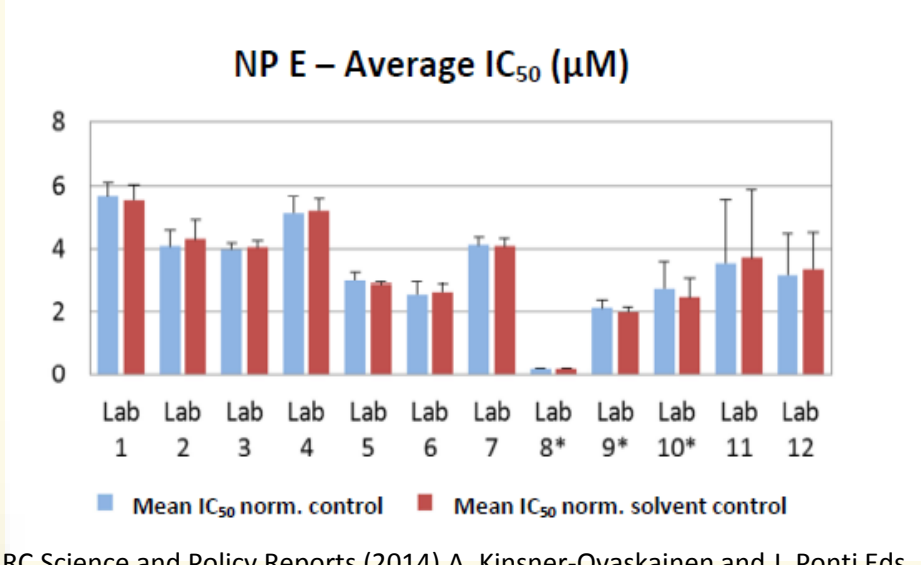


TiO_2 cytotoxicity measured by Neutral Red Uptake assay: the increase in optical density at the higher concentrations is also highlighted in absence of cells

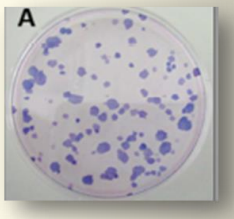


Critical aspects : cytotoxicity assays

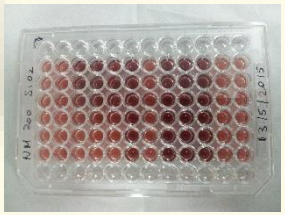
To date the most reliable and reproducible cytotoxicity assays for NMs are:



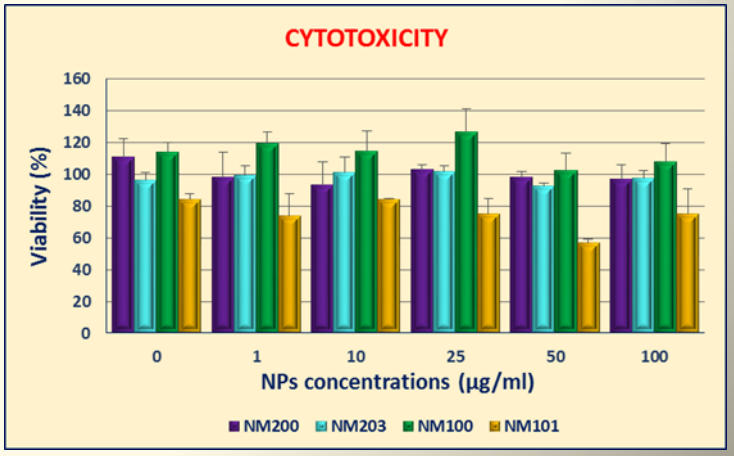
JRC Science and Policy Reports (2014) A. Kinsner-Ovaskainen and J. Ponti Eds



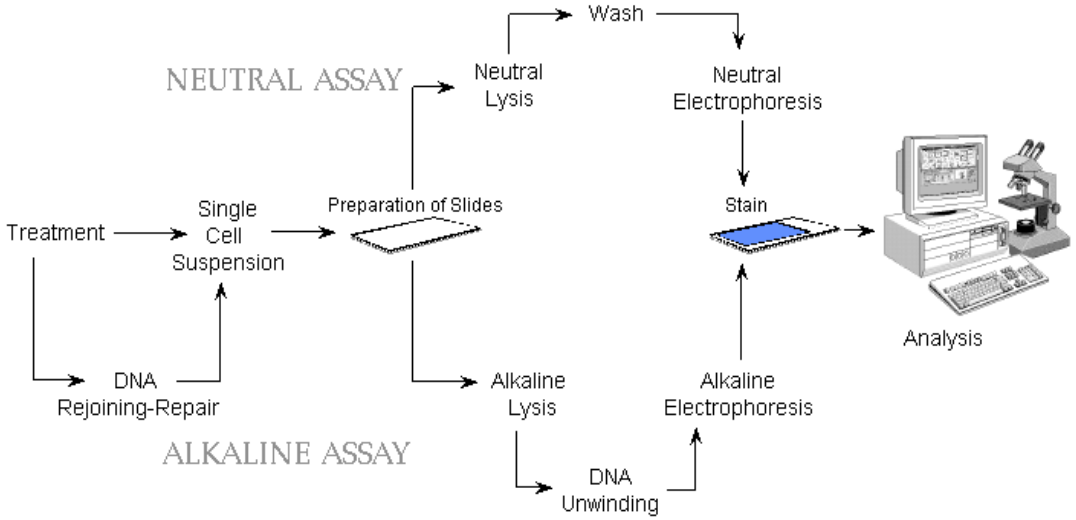
CFE

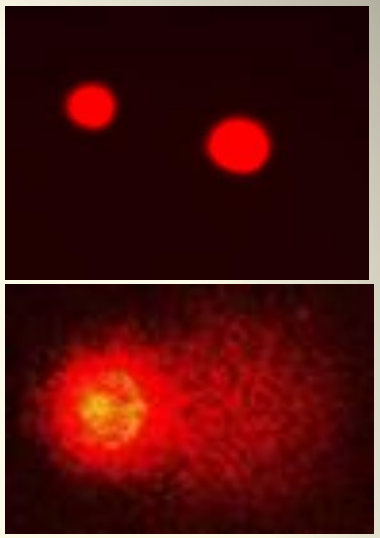


MTS



Comet assay





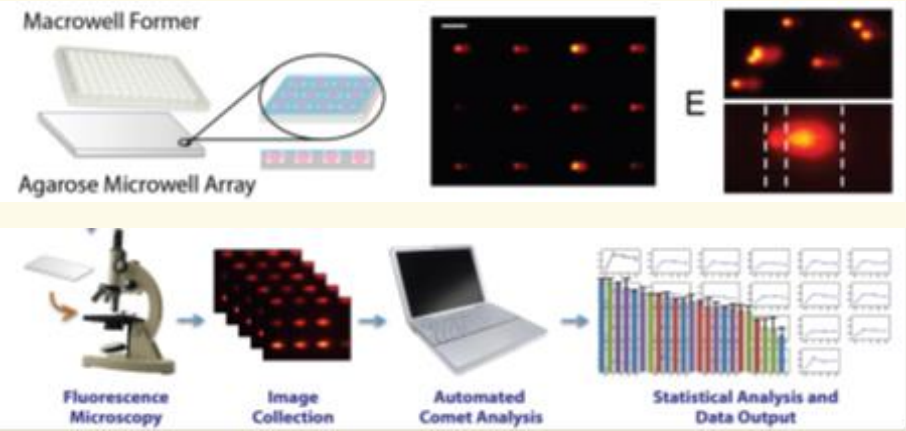
Advantages

- DNA damage is measured at single cell level
- Applicable on many cell types
- *In vivo* and *in vitro* tests
- No *in vitro* cultivation step required
- Cheap
- Fast
- Simple
- Applicable for NMs evaluation

Critical points

- The detected DNA damage does not correspond to fixed mutations
- No OECD guidelines available for *in vitro* testing
- Possible interaction of NMs in some steps of the protocol

High-Throughput Comet assay



Advantages

- allows to test a large number of NMs at different concentrations on different cellular models
- reduces the inter-experimental variability
- reduces cost and time
- allows to bild up a database for NMs hazard ranking

Critical points

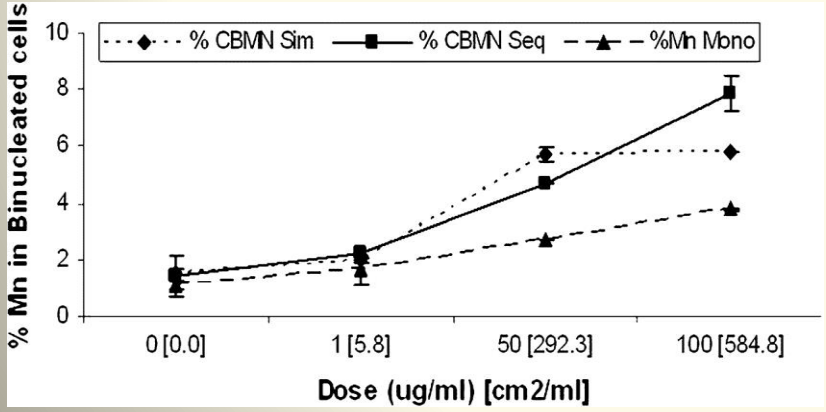
- validation is needed
- visual scoring is time consuming
- automatic scoring is very expensive

OECD genotoxicity guidelines: critical aspects

WPMN Workshop on the Genotoxicity of Manufactured Nanomaterials 18-19 November 2013 Ottawa, Canada

Consensus statements:

- ✓ The use of the Ames test (TG 471) is not a recommended test method for the investigation of the genotoxicity of nanomaterials.
- ✓ The test guidelines program should consider modification of the *in vitro* micronucleus assay to recommend, where cyto B is used.



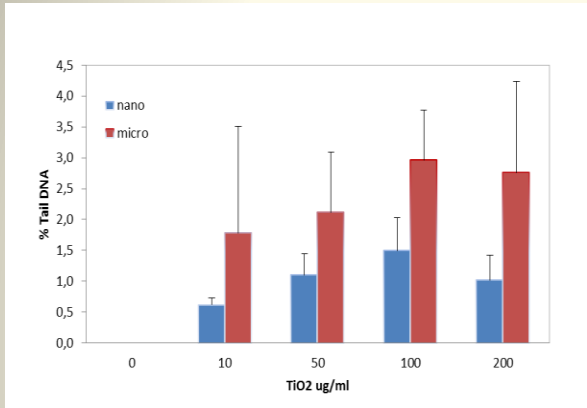
S.H. Doak et al. Mutagenesis 2009;24:285-293

Cytochalasin B treatment may modify results of the test

Recommendation: apply a protocol to take into account Cytochalasin B endocytosis inhibition

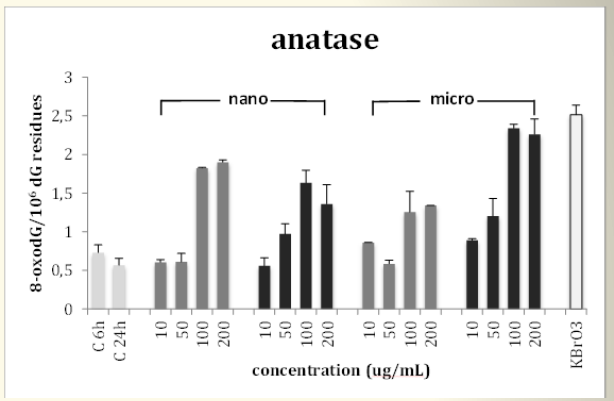
Genotoxicity evaluation of NMs: experience at ISS

Titanium dioxide genotoxicity in peripheral blood mononuclear cells

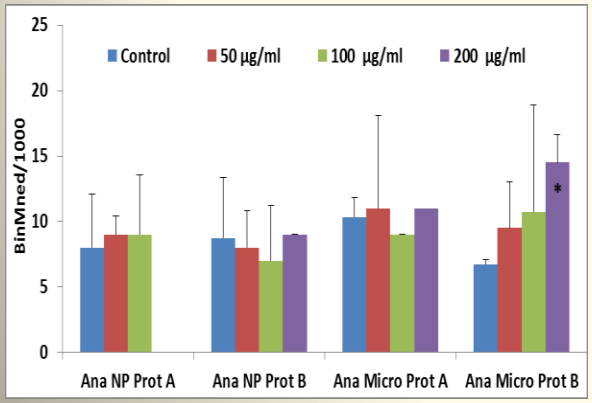


Comet assay

Oxidative DNA damage by comet assay and 8-OxoG are measured in PBMCs (lymphocytes /monocytes)



8-OxoG detection



Micronucleus assay

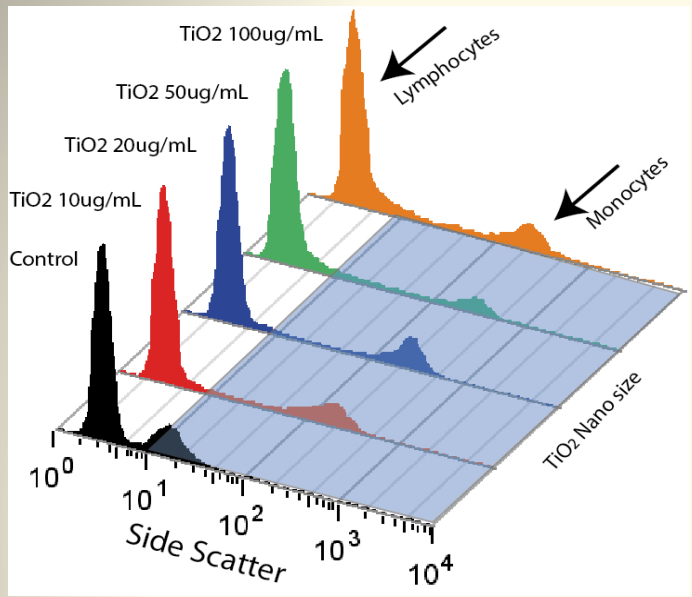
Micronuclei are analyzed in proliferating lymphocyte subpopulation

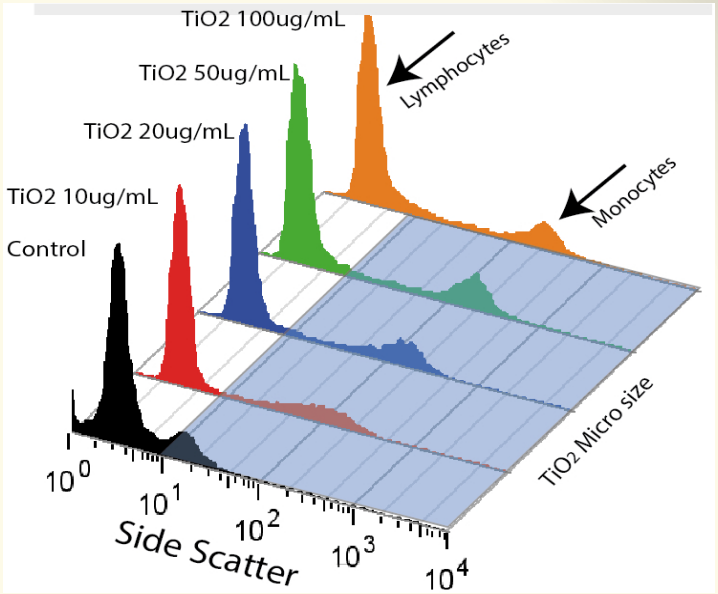
Differences may be due to assay or intrinsic cell population sensitivity and/or in DNA damage repair capacity of proliferating *vs* resting cells

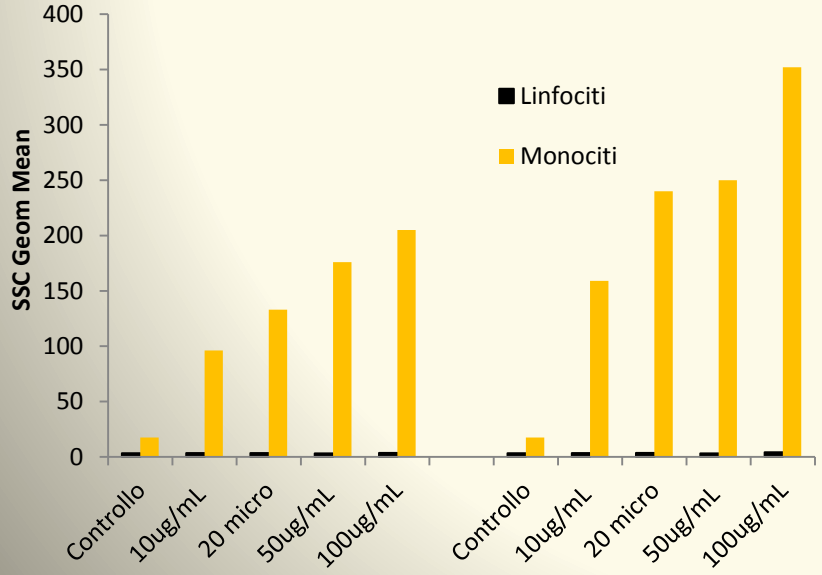
Andreoli et al. Manuscript in preparation

Nanoparticle uptake: not all cells can do it

In collaboration with Dr G. Leter- ENEA Casaccia

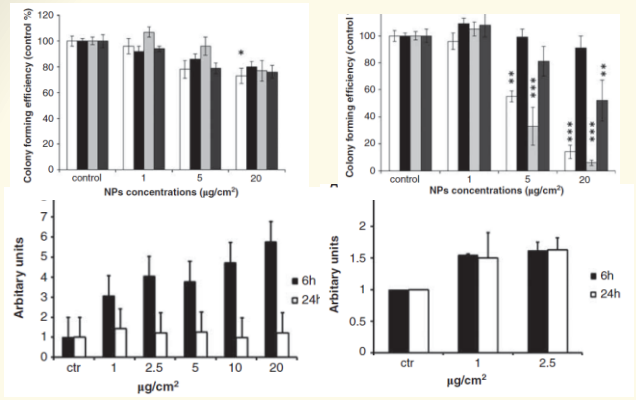






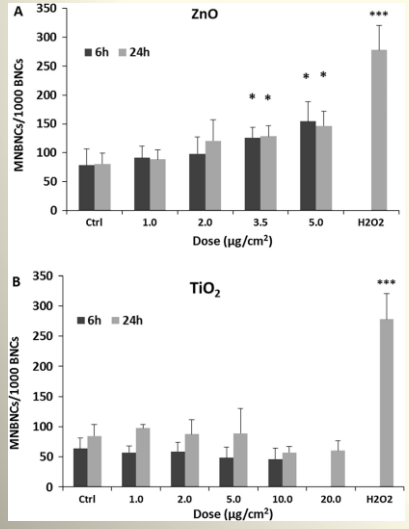
The negligible uptake of NPs by lymphocytes can determine the absence of response observed in the micronucleus assay

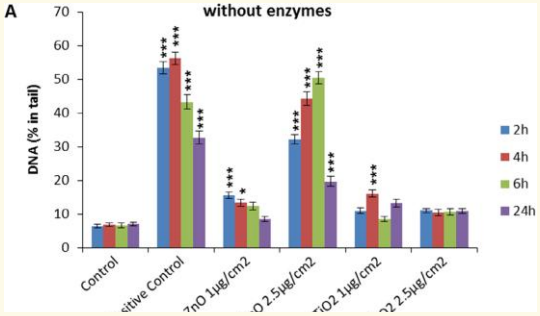
Oxidative damage and genotoxicity in Caco-2 cells: comparison TiO_2 and ZnO

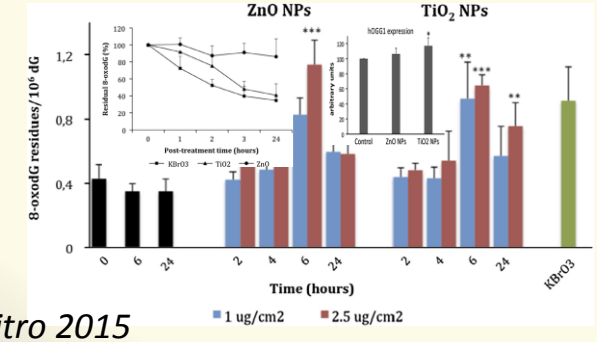


TiO_2 **ZnO**

De Angelis et al: Nanotoxicology 2012







Zijno et al: Toxicology in Vitro 2015

ZnO produces strong citotoxicity

Both NMs induce ROS but most of ROS produced by TiO_2 was removed after 24 hours

Only ZnO induces micronuclei

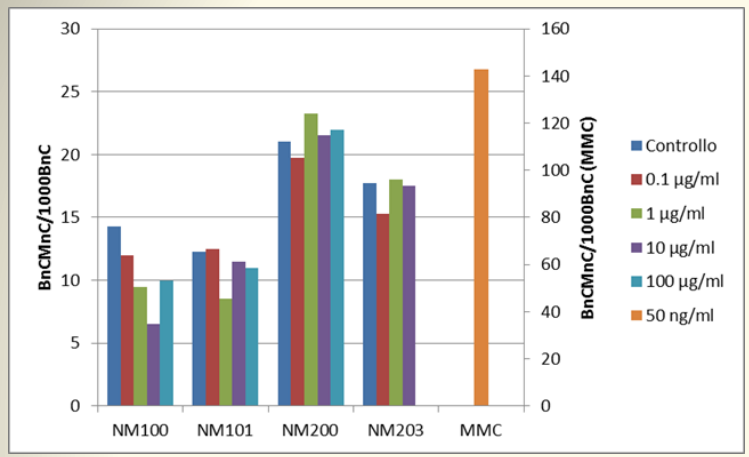
Only ZnO induces DNA damage detected by Comet assay

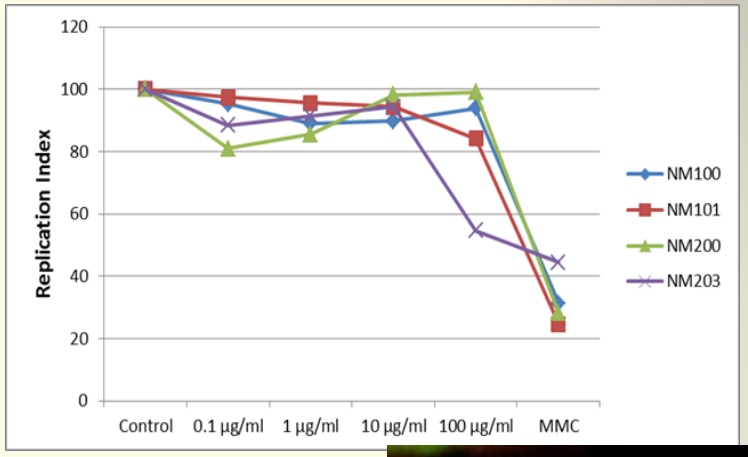
Both NPs induce 8-OxoG but only ZnO produces persistent 8-OxoG.

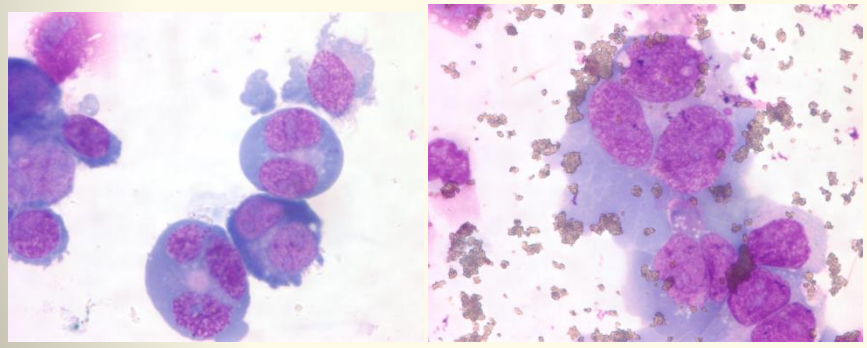
Increased expression of OGG1 is observed only by TiO2 treatment

ROS production and oxidative DNA damage are not sufficient to trigger a genotoxic effect

Micronucleus analysis in the framework of Nanoreg project

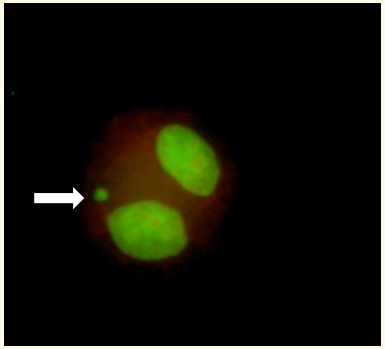




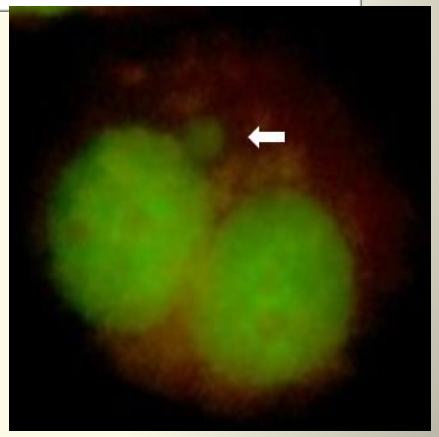


Untreated lymphocytes Giemsa staining

TiO2 treated lymphocytes Giemsa staining



Untreated Beas-2B cells. AO staining



TiO2 treated Beas-2B cells. AO staining

Titanium dioxide NPs may interfere with the visual scoring. Cytofluorimetric micronucleus analysis may represent a suitable alternative

In vitro assays for immunotoxicity studies

Key points

- Definition of measurable and predictive endpoints
- Choose of the immunological target (innate or adaptive responses)
- Selection of representative functional assays (feasibility, reproducibility)
- Use of reliable systems (*ex vivo* human or animal cells/tissues, cell lines)
- Set up of assay conditions (dose selection/metrics, exposure, time points)
- Characterisation of the NMs at different steps of the assays

- *Session TS.III.E Nanotoxicology meets green chemistry: toward safe and sustainable nanomaterials (part II 15:00 – 16:30)*
- TS.III.E.3 Nanoimmunotoxicity: *in vitro* and *in vivo* approaches (*Gabriella Di Felice, ISS)*

Summary and Conclusion

Some efforts are still needed to adapt tests for the *in vitro* assessment of NMs safety

Main issues to be solved:

- Standardization of procedure to prepare NMs
- Standardization of procedure to characterize NMs after suspension in culture medium
- Definition of appropriate methods to evaluate cytotoxicity and genotoxicity
- Choice of most appropriate cell systems
- Choice of most suitable protocols

Acknowledgements

Cristina Andreoli
Flavia Barone
Isabella De Angelis

Dept. Environment and Primary Prevention

Gabriella Di Felice

Dept. Infectious, Parasitic and Immune-mediated Diseases

THANK FOR YOUR ATTENTION

Critical aspects: immunotoxicity assays

General critical aspects:

- ✓lack of systematic data on immunological effects
- ✓inadequate knowledge of the mechanisms involved in widely diverse effects
- ✓absence of dedicated guidelines
- ✓need to develop a proper assessment of immunological/immunotoxic effects for regulatory purposes (not necessarily the same!)

Technical critical aspects:

- ✓agglomeration / aggregation NM
- ✓changes of NM due to the culture system
- ✓NM interference with the assays (optical density)
- ✓biocompatibility (" carry-over " of the solvents)
- ✓choice of the population /cell line (human / murine?)
- ✓contaminants with biological/immunological activity (LPS , endotoxin)
- ✓ limited relevance for risk assessment

Nanomaterials hazard identification/characterization

In vitro tests fulfill a primary role in the hazard assessment for NMs' safety

In vitro vs in vivo approaches

Advantages

- Ethically acceptable
- Simplicity (facilities)
- Shorter
- Economic
- Mechanisms

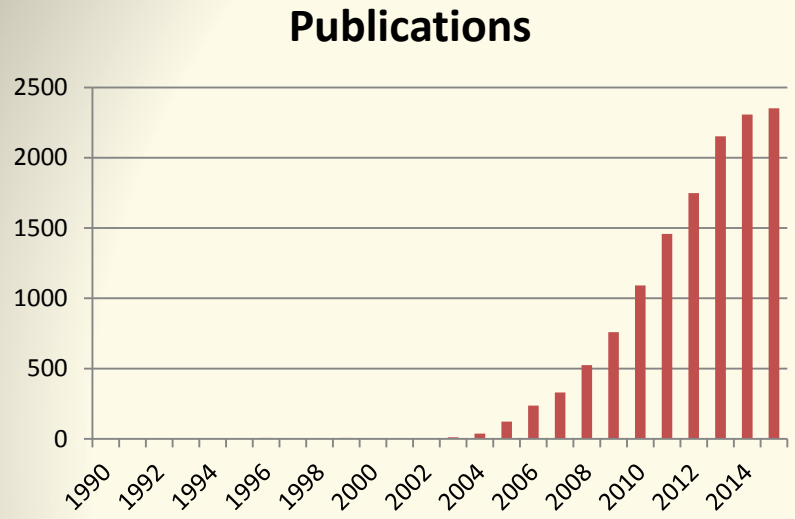
Critical points

Cell behaviour in culture and in the whole organism is different (the coordinated tissue response)

Ideally

A combination of *in vitro* tests simulating as closely as possible *in vivo* situation

Critical aspects in NMs safety assessment



PubMed keywords: nanomaterials AND toxicity
Search results (10 August 2016): 13583 articles

Poor quality and reproducibility of experimental data prevent a reliable comparison among studies and the assessment of safety of tested NMs

Basic questions:

- Is the nanomaterial safe?
- What properties influence or modify its toxicological profile?
- What is the underlying mechanism of toxicity?

Pitfalls:

- Suspension procedure
- Phys-Chem characterization
- Biological experimental models
- Interference with tests
- Experimental protocols
- Contaminant with biological/ immunological activity

Comet assay: critical aspects

Possible NM-comet assay interactions during assay performance:

- NMs present in or in contact with cells may induce additional breaks in “naked DNA”
- Photocatalytic NMs present in the nucleoid may induce additional breaks
- Particles may interfere with the scoring possibly reducing head intensity
- NMs associated with nucleoid DNA may lead to less migration under electrophoresis
- NMs in the nucleoid may interfere with the action of enzymes during the modified protocol of comet assay