Nanotoxicology
Nanoparticles & Human Health

Journée Annuelle du LabEx NanoSaclay, 13 janvier 2022

DE LA RECHERCHE À L'INDUSTRIE

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ISO definition:
ISO/TS 80004-2:2015
One dimension between 1 and 100nm

EU precision:
2011/696/EU
At least 50% of particle (in number) are nano

Up to 40 definitions depending on domain of expertise, country…

Nano-objects
- Hard and software to visualize et modelize
- *atomic force microscopy, lithographic nano-printing,...*

Nano-materials
- nanoscale structures in raw format
- *nanoparticles, nanotubes, quantum dots,...*

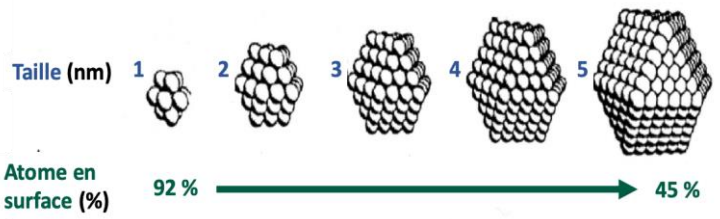
Nano-intermediates
- Unfinished products with nano properties
- *coating, contrast medium, optical compounds,...*

Nano-containing products
- finished products containing nanotechnologies
- *cars, textile, painting,...*

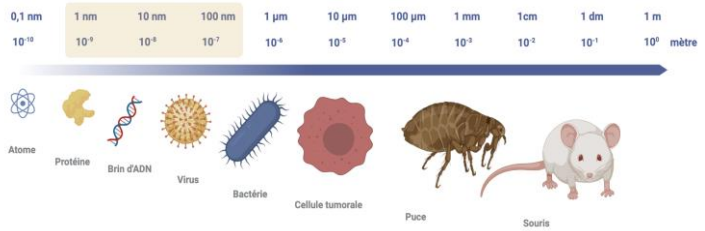
→ Communication
→ Effect description and attribution

…

Surface effects

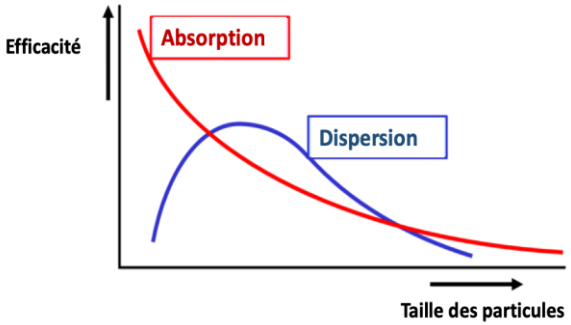


Level of life

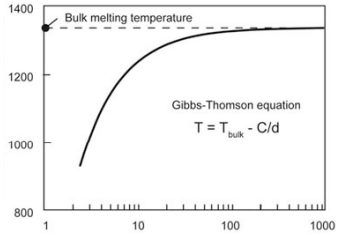


Quantic effects

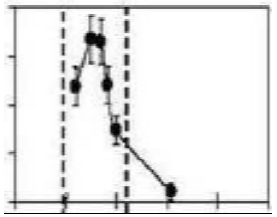
Optical properties



Fusion point



Catalytic activity



NP diameter (nm)

Interesting new properties but… safety concerns

Experience of Ultrafine particles and asbestos toxicity

Increase reactivity

Every new nano has to be considered as unique

→ Needs for characterization

→ Need to demonstrate absence of toxicity prior using

Characterisation of nanoparticles

What they are

Chemical identification
- Composition,
- Impurities

Physical descriptors
- Size
- Shape,
- Porisity
- Surface area

Computational descriptors
- Agregation tendancy

Where they go

Stability
- Chemical (solubility)
- Physical (agglomeration)
- Thermodynamic (phase transition)

Surface charge

Surface reactivity

Bio-Nano interface

Mobility in different media

Toxicokinetic and uptake

What they do

Physical hazards
- Flammability,

Human toxicity (acute/chronic)
- Cytotoxicity
- Genotoxicity
- Oxidative stress
- Inflammation

Ecotoxicity (acute/chronic)
- Cytotoxicity
- Genotoxicity
- Oxidative stress

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Physico-chemical properties of manufactured nanomaterials - Characterisation and relevant methods. An outlook based on the OECD Testing Programme

Kirsten Rasmussen[a,*], Hubert Rauscher[a], Agnieszka Mech[a], Juan Riego Sintes[a], Douglas Gilliland[a], Mar González[b], Peter Kearns[b], Kenneth Moss[c], Maaike Visser[d], Monique Groenewold[d], Eric A.J. Bleeker[d]

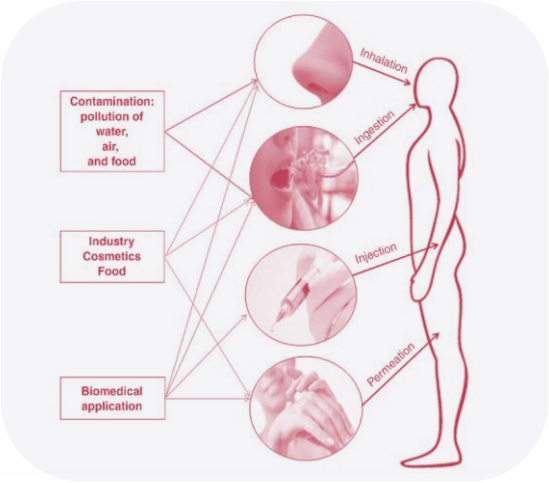
Characterization of

- **Physico-chemical**
- **Behaviour**
- **Hazard**

Factors influencing NM toxicity

- **Size – Surface Area**
- **Surface characteristics**
- **Shape**
- **Cristallinity**
- **Stability**
- **Impurities**
- **…**

+ Route of exposure



Crisponi et al, 2017

→ **Essential parameters for NP characterization in toxicological assays**

1. Chemical composition
2. Size distribution
3. Agregation / Agglomeration state
4. Shape
5. Surface Area
6. Surface structure
7. Surface potential
8. Solubility /Dispersibility

ISO/TR 13014:2012

Direct and indirect methods

Different methods for 1 parameter: can bring to conflictual data

Polydisperse sample with DLS or MET for hydrated samples

Approaches for risk evaluation

Epidemiological studies

- Large cohorts for statistic robustness
- Quantification of exposure + delay

Toxicological studies

- Description of mechanisms
- Anticipation of exposure

Risk = **Exposure** x **Danger**

Risk management

Metrology

Hazard effect of a given compound
Evaluation of the toxicity
Support for legislation

Nanotoxicology: study of adverse effects of engineered NM/NP on living organisms

Understanding of NP toxicity is superficial

- Most of the studies are mechanistical not toxicological
- Though comparision of published results
- Unique nature of NP and poor characterization

Cell model
Test procedure
NP and characterization
Dose and time of exposure
Results interpretation

Join the dialogue

The nanotoxicology community should implement guidelines on the types of information that are required in their research articles to improve the quality and relevance of the published papers.

Editorial, Nat. Nanotechnol. 2012

Mechanism of nanotoxocity

- **Intrinsic properties of NP**
  surface area -> surface effect
  presence of metals -> reactivity

- **Activation of intracellular pathways**
  Enzymatic systems

Oxidative Stress production
Protein oxidation
Lipid peroxydation
DNA damage
Mitochondrial dysfonction

Genotoxicity
Direct or indirect damage

Inflammation
Activation of MAPK or. NFkB signalling pathways

Cytotoxicity
Reduced rate of proliferation
Apotosis
Consequence of direct damage to membrane, mitchondria, lysosome, cytoskeleton

Conventional approaches are restricted to 1 or 2 endpoints

→ omics for hight throughput analysis

Proteomics: Identification of biomarkers or toxicity signature

Genomics: Identification of mode of action or sublethal alterations

Interferences with toxicity assays

NM can adsorb or modify assay reeagent

- **High adsorption capacity**
- **Hydrophobicitiy**
- **Surface charge**
- **Catalytic activities**

modification of structure
fonction
concentration

NM can absorb or scatter light

- **Optical properties**

modification of absorbance
luminescence
fluorescence

Misintrpretation
Artefact

→ Adaptation of protocols
- Extensive washes
- Test replacement
- Supernatant transfer
- Gating adaptation

→ Use of appropriate controls

intrinsic fluorescence or absorbance of NP
NP + assay component
NP + analyte
…

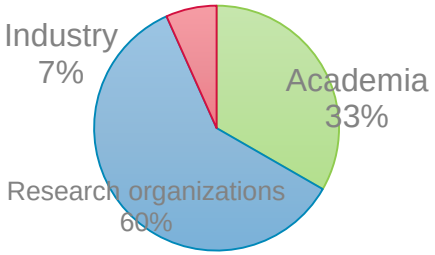
→ Definition and use of reference materials

Quality of testing: an interelaboratory comparision study

Nelissen et al., Nanomaterials, 2020

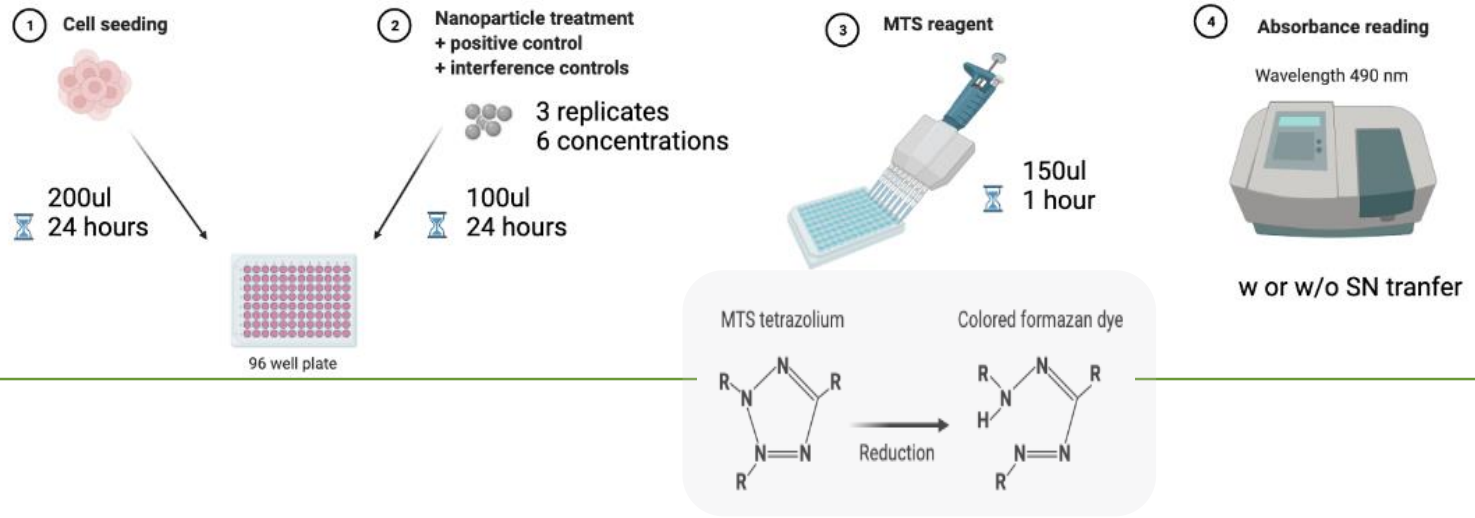
Interlaboratory comparison study within 15 europeans labs

→ Cytotoxicity assay on 2 characterized NM
PS-COOH 50 nm (-)
PS-HN2 40 nm (+)



Sharing of
- SOP: standard operating procedurre
- cells: identical frozen cell stock of A549 alveolar epithelial cell line
- serum: central stock prepared at 1 location
- nanoparticle: stable dispersions in cell medium

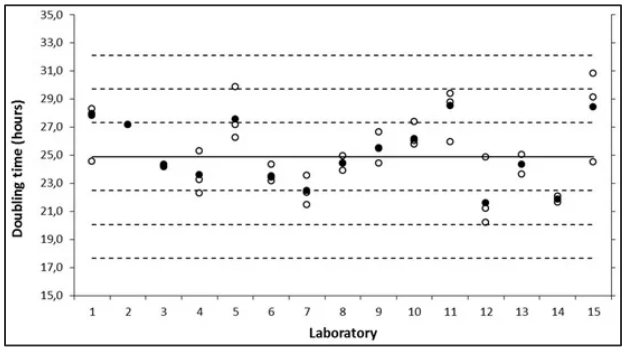
MTS assay protocol



+ best practice training impact on MTS assay results and reproducibility

Nelissen et al., Nanomaterials, 2020

Course of the study



- **Tier 1** Cell growth rate and viability

  → confirm the lab proficiency in their cell culture performance

- **Tier 2**: MTS assay following SOP

  → measure the inherent performance for the test
  → exhibit a large variability in results
  → notification of optical interference
  → highlight critical phases

Protocol Step	Critical Phase
All steps	- Verification of pipets and instruments - Use of single vs. multi-channel pipets - Pipetting technique - Adherence to timings stated in the SOP
Preparation and storage of staurosporine stock	- Dissolution of lyophilized product
Preparation of staurosporine working solution	- Low pipetting volume
Preparation of NP dilutions in cell culture medium	- Low pipetting volume - Dispersion protocol
Preparation of dosing plate	- Different pipetting volumes
Plating cells	- Use of antibiotics-free cell culture medium - Cell counting method - Homogeneous suspension of cells - Edge effects
Exposure to test item	- Removal of medium from cultures - Homogeneous suspension of test items - Application of test solutions onto cultures
MTS assay	- Removal of medium from cultures - Air bubbles - Precipitate of MTS reagent - Transfer of MTS reagent for read-out - Spectrophotometer specifications

- **Tier 3**: MTS assay following a training phase

  → improve reproducibility

→ Degree of complexitiy related to good practice in nanosafety
→ Training and SOP improve the quality of results
→ Needs for harmonized protocol and good practice

Regulatories strategies

Grouping: arrangement of NM into groups based on common attributes
→ groups relevant to risk: physico-chemical properties + exposure data
→ more convenient fot chemicals rather NM

Ranking: assigning a position in a scale from high to low hazard according to reference

Read-across: use relevant information forrm analogous substances to predict properties of the target material

Safe by design: toxicity mitigation strategies
→ identification of features making NM potentially toxic
→ evaluation of the correlation between desired properties of NM and their features
→ design of the synthesis strategy for paramters modulation (release, accumulation, bio-accumulation)

NanoReg2	CATEGORIZATION					
	NM100	NM101	NM102	NM103	NM104	NM105
A549	Non Toxic	Non Toxic	Non Toxic	Non Toxic	Non Toxic	Non Toxic
Caki-1	Slightly Toxic	Slightly Toxic	Toxic	Non Toxic	Non Toxic	Slightly Toxic
Hep3B	Slightly Toxic	Slightly Toxic	Toxic	Slightly Toxic	Toxic	Toxic
Calu-3	Slightly Toxic	Non Toxic	Non Toxic	Non Toxic	Slightly Toxic	Toxic
Caco-2	Non Toxic	Slightly Toxic	Slightly Toxic	Non Toxic	Non Toxic	Slightly Toxic

R. Grall

→ Importance of biological model on conclusion

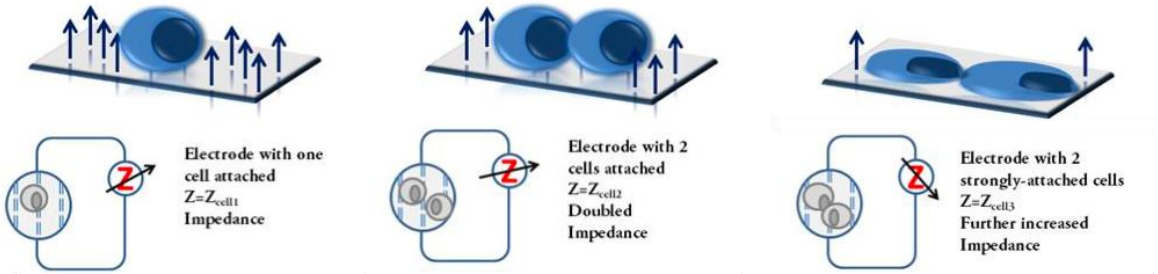
X-CELLigence system for cellular impedence measurement

- 6x96 well-plates approach
- Real time
- Long term
- Non destructive

Measure every 5 minutes up to 15 days

Cell index

Dimensionless parameter
Relative change in electrical impedence



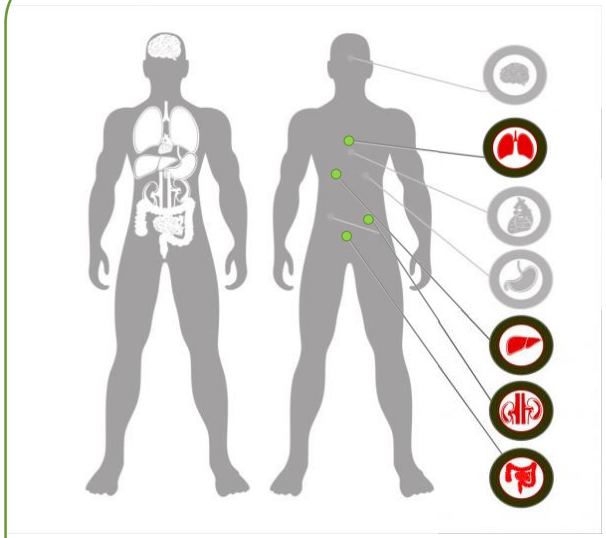
Cell number
Cell proliferation
Cell death
Cell size/shape
Cell-substrate attachment quality
Membrane potential

→ Multiparameter analysis for a global response
→ Information of time and dose of interest
→ No interference until now

Study design

Impedence measurement every 5 min for 6 hours
every 10 min for 5 days

5 human cell lines



15 nanoparticles

6 TiO_2
3 SiO_2
3 ZnO
Ag
$BaSO_4$
CeO_2

5 doses

0,6 µg/cm²
6 µg/cm²
16 µg/cm²
32 µg/cm²
64 µg/cm²

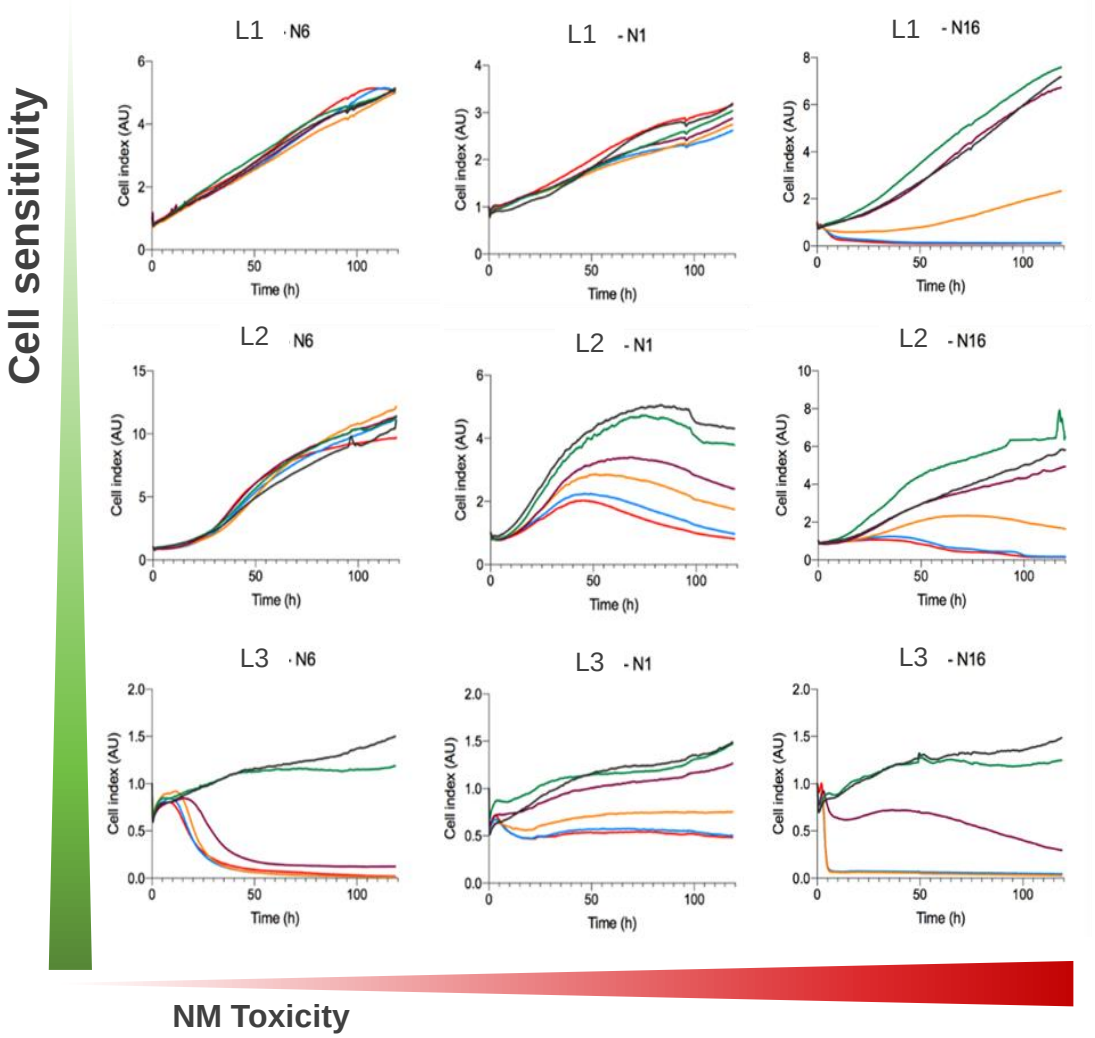
~~Single cell model~~ ~~Unique NP~~ ~~Unique dose~~

→ no general conclusions (different genetic background, differenciation state, function)
Different sensitivity (and uptake ?)

→ adaptation of statistical analysis tool developed for omics management

Grall et al., in preparation

Cell index profiles



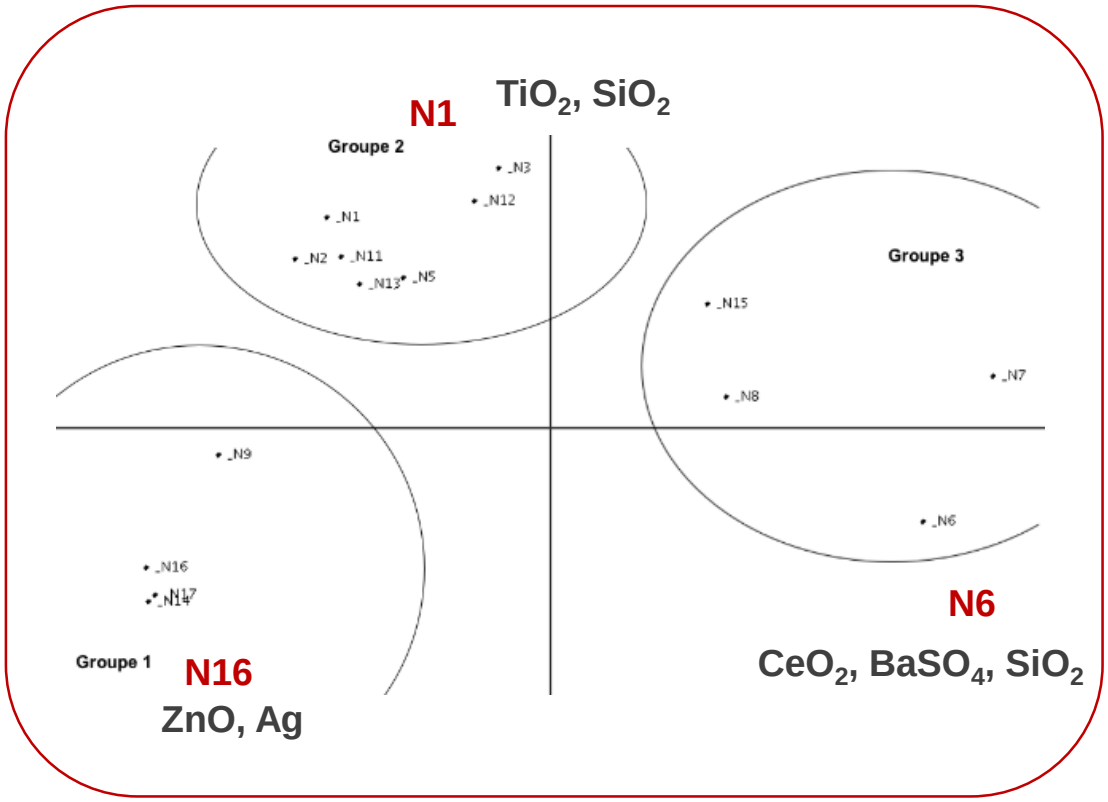
Grall et al., in preparation

Hierarchical clustering → Classification + grouping

Group 1: highly cytotoxic
decrease of cell index
at all doses (L3)
at highest doses (L1)

Group 2: Intermediate cytotoxicity
decrease of cell index
intermediate (L3)
moderate (L2)
no modification (L1)

Group 3: Low cytotoxicity
decrease of cell index
(L3)
slight (L2)
no modification (others)



→ Every NM of the same composition were found in the same group
→ Ag and ZnO are known to be toxic…
→ Exception with SiO_2 in two groups but with different stability profiles
(the mid toxic is highly soluble → dissolution could influence the tox profile)

Grall et al., in preparation

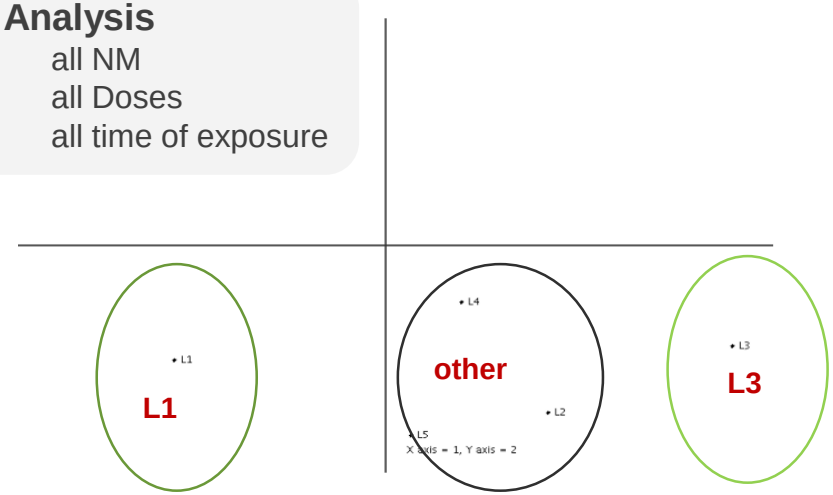
Influence of cell line

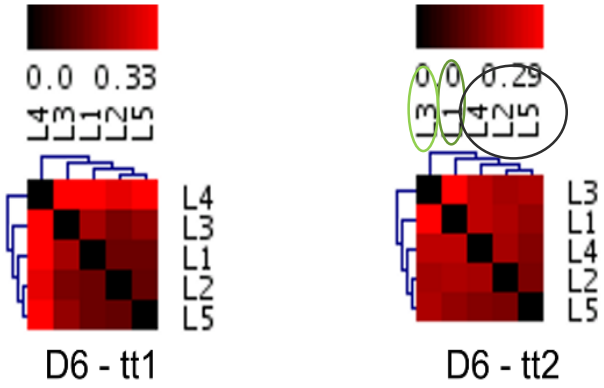
Guidelines with most appropriate - cellular model,

- time of exposure,
- dose range

PCA Analysis

all NM
all Doses
all time of exposure





The longest time is more accurate in grouping

→ Analysis of 15 NM on only **L1** could lead to a conclusion where almost **none of them are toxic**

Most resistant cell line
But also widely used for toxicity studies
as - representative of airway epithelium (major route of exposure)
- immortalized cells with reproductible results

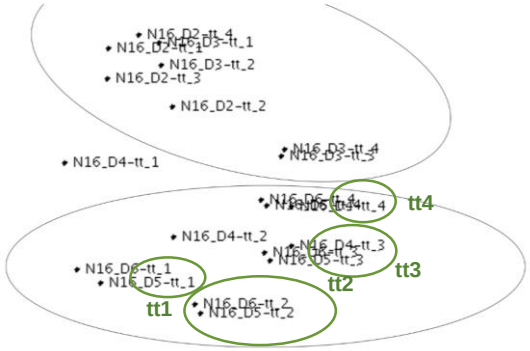
→Analysis of 15 NM on only **L3** would have the opposite conclusion: **every NM are toxic**

Grall et al., in preparation

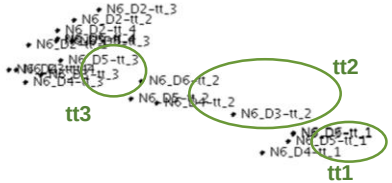
Influence of dose

Guidelines with most appropriate - cellular model,
- dose range
- time of exposure,

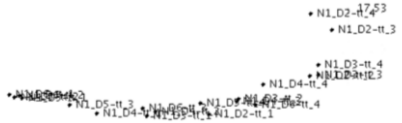
N16 (Group 1)



N6 (Group 2)



N1 (Group 3)



→ **Spatial organization**
D2 and D3 at all times
D4, D5 and D6 at all time

Subgroup by time of exposure

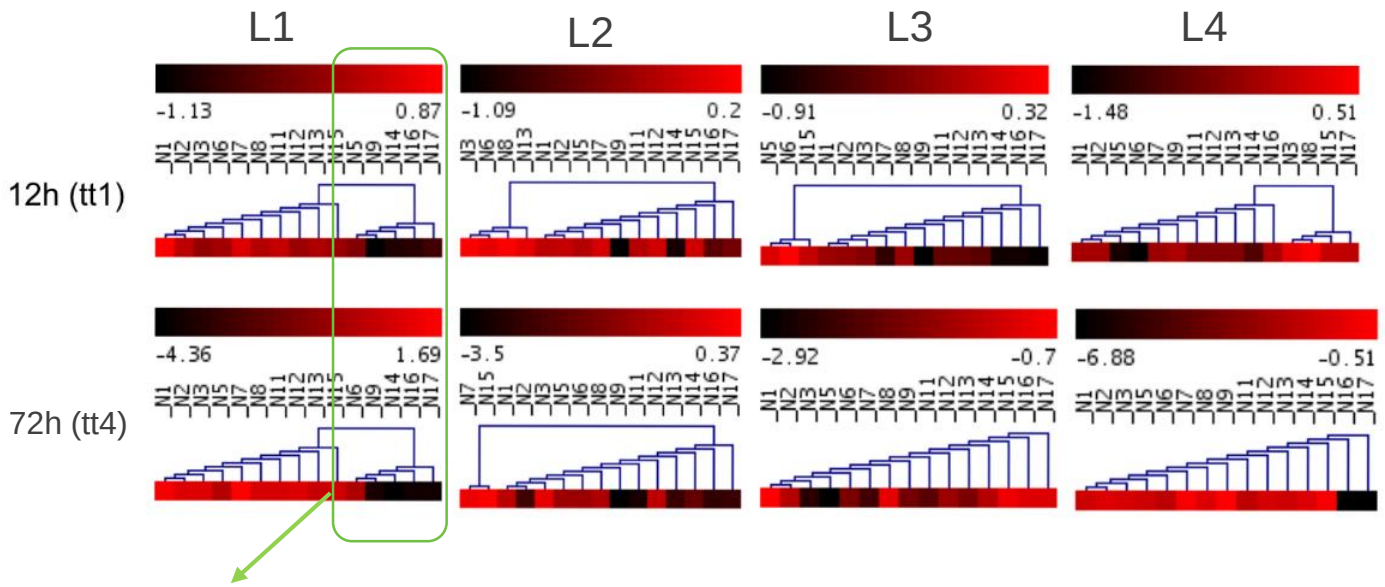
→ **Weak spatial organization**

→ **No spatial organization**

→ Absence of impact of dose on classification for low toxic NM

Grall et al., in preparation

Guidelines with most appropriate - cellular model,
- dose range
- time of exposure,



N9
N14 Higly toxic NM
N16 **No influence of time**
N17

tt1 → tt4
Multi group organization to Hierarchical classification
Different kinetics between NM

→ No influence of exposure time for both toxic NM and/or resistant cell line

Grall et al., in preparation

Study conclusion

➡ Use of HTS methodology for multiparameter analysis

➡ Use of a panel of at least 3 different cell lines
With sensitivity evaluated prior to study (reference materials)
With origin relevant in view of potential organ of exposure or retention
→ more accurate hazard information by minimizing internal sensitivity

Resistant	**Mid sensitive**	**Sensitive**
Short time exposure	Several times of exposure increase accuracy	Several times of exposure increase accuracy

➡ Use of fully characterized NM prepared according SOP

➡ Use of several time of exposure (24h is not enough)
to integrate different kinetic of toxicity profile
to integrate nanoparticle modification like dissolution, sedimentation…

Take home message

No clear conclusions about hazard

- Lack of experimental process harmonization
- Need for a relevant and powerful methodology

→ **Need of HTS methods that sticks to guidelines**

Impedence measurement is an approach that could fill the gap of knowledge

- Set appropriate experiments to avoid interferences
- Positioning with regards to existing experiments for toxicology
- Importance of time of exposure : the longer the better ?

→ **Choice of right conditions for further relevent experiment**

Identification of critical parameters:

- Interferences
- Choice of models
- Positive and negatve particle of reference for Intercomparative studies

→ **Lack of knowledge towards NP interaction with biology**

Research priorities relevant to development or updating of nano-relevant regulations and guidelines

Methods

- ❑ Clear definitions
- ❑ Methods for differenciation of primary NM, Agregates, Agglomerates
- ❑ Standardized methods for NM preparation
- ❑ Standardized methods for NM suspension in different media
- ❑ Mechanisms definition
- ❑ Methods for exposure dose measurment
- ❑ Relevant short term hazard in vitro/in vivo evaluation
- ❑ Long term hazard models
- ❑ Methods to test transformation of NM through life cycle
- ❑ Validation + standardization of methods for characterisation
- ❑ Identification of exposure/hazard relevant dose metrics
- ❑ Inventory of validated and standardized tool

reference materials
SOP
toxicity + factors
harmonization + standard metrics
HTS
biomarkers
Common programms
Decision tree

Data

- ❑ Identify characteristics that influence release
- ❑ Identify characteristics that influence exposure

Strategies

- ❑ Refinement of NM definition
- ❑ Grouping and Read across strategies
- ❑ Developpement of risk mitigation strategies (Safe by design)

Merci de votre attention

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