NEED FOR GUIDELINES SPECIFICALLY ADAPTED FOR THE TOXICITY TESTING OF NANOMATERIALS

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INTRODUCTION

- Nanomaterial production is increasing each year
- Nearly anything can be toxic at a high enough dose,
  - How toxic are nanomaterials at the potential concentrations at which they might be used?
- Any toxic effects of nanomaterials will be specific to:
  - Type of base material
  - Size
  - Shape
  - Coatings
- Nanotoxicity studies are focused on local effects
  - Each research group uses different
    - cell lines
    - culturing conditions
    - incubation times

INTRODUCTION

- Best strategy to tests nanoparticles? No concerns

- **How are we testing nanomaterials?**
  - Standardized protocols for standard chemicals to assess the hazards of substances released into the environment
  - Not always appropriate for nanomaterials → misleading effects

MODIFICATIONS OF SUCH PROTOCOLS ARE REQUIRED FOR NANOMATERIALS

- **OECD**
  - Spearheading a coordinated strategy focusing on an initial selection of nanomaterials and characterization properties

- **European Commission**
  - Seventh Framework Program (NMP.2012.1.3-3 Regulatory testing of nanomaterials)
  - Developing a way of standardized and puting an order on the nanomaterial world
  - QSAR ideas

- **Why is it so problematic to define some protocols for NPs?**
  - Differences in size, shape, coating and chemical nature increase the difficulty level for a unique, linear and coherent understanding of the results.
  - Lack of Reference materials:
    - REFNANO
    - ERDC-NIST workshop on nano-silver
    - NIST (National Institute of Standards and Technology, USA)
    - JRC-IRMM (Joint Research Centre—Institute for ReferenceMaterials andMeasurements, European Commission)

Table 1
Properties to characterise nanomaterials in media (stock solution) proposed by a range of authors.

Property	Oberdorster et al. (2005)	Powers et al. (2006, 2007)	Thomas et al. (2006)	Warheit (2008)	Klaine et al. (2008)
Size distribution	*	**	**	**	**
Agglomeration state/dispersion	*	**	*	**	*
Crystal structure	*	*	*	**	
Chemical composition	*	*	*		**
Surface area and Porosity	*	**	*	**	**
Surface chemistry		**	*	**	*
Surface charge		*	*	**	*
Shape and morphology		**	**		*
Dissolution/ Solubility		*	*		**
Physical/chemical properties (purity)		**		**	
Methods of synthesis				**	

*: Of importance; **: Priority.

- Lack of characterization in bibliography
  - Essential to compare results and conclude
  - How it is necessary to characterize?
- Several possible classifications for nanomaterials
- Difficulty for tracing and quantifying some nanomaterials within organisms or environment
  - -Need of labeling
- Toxicity studies of NPs use:
  - -Different cell lines
  - -Different incubation times
  - -Different range of concentration
  - -...

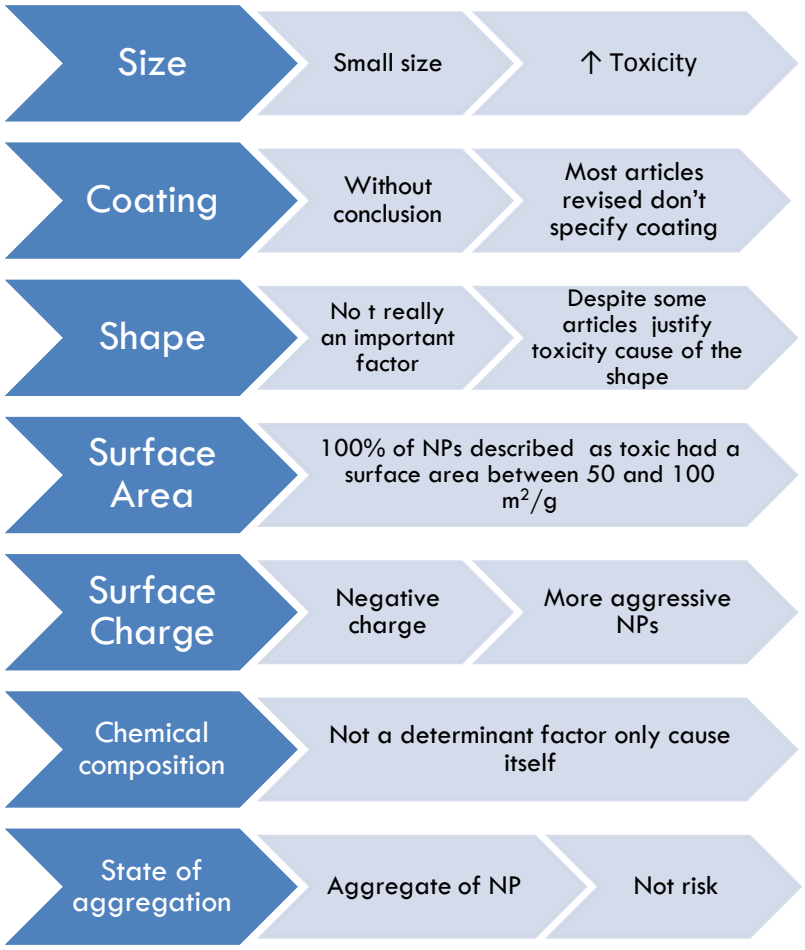
NANOSARS

Hypothesis: The behavior of NPs is different according to its intrinsic properties

It should be possible to predetermine a model of toxicity according to these characteristics

Toxicity NPs	Size						Coating				Morphology / Shape						Surface Area (m2/g)					Surface charge			Chemical composition			Dissolution		Aggregation		Magnetic	
	< 4 nm	5 - 19 nm	20 - 49 nm	50 -99 nm	100 - 249 nm	> 250 nm	No coating	Aminosilane Thiolsilane	Gold	RE (rare earth)	Round/Spherical	Equiaxial	Irregular	Cubic / crystal	Rod	Tubular	< 10	10 - 25	25 - 50	50 - 100	> 100	Neutral	Cationic / Positive	Anionic / Negative	C	Metal oxides	Metals	**PBS**	**Aqueous** / Deionised water	Yes	No	Paramagnetic	No magnetic
Tox 1	16	24	24	24	12	0	88	4	4	4	68	0	0	5	23	5	0	33	33	0	33	0	95	5	4	36	60	33	67	100	0	96	4
Tox 2	7	21	36	14	21	0	100	0	0	0	43	7	21	14	0	14	20	20	30	10	20	0	100	0	25	63	13	0	67	60	0	75	25
Tox 3	14	0	43	29	0	14	100	0	0	0	50	25	0	0	0	25	20	20	40	0	20	50	50	0	14	43	43	0	75	50	0	71	29
Tox 4	60	0	40	0	0	0	100	0	0	0	60	0	0	20	0	20	0	0	0	100	0	0	50	25	20	20	60	0	100	0	100	20	80

NANOSARS


Size
Small size
↑ Toxicity
Coating
Without conclusion
Most articles revised don't specify coating
Shape
No t really an important factor
Despite some articles justify toxicity cause of the shape
Surface Area
100% of NPs described as toxic had a surface area between 50 and 100 m^2/g
Surface Charge
Negative charge
More aggressive NPs
Chemical composition
Not a determinant factor only cause itself
State of aggregation
Aggregate of NP
Not risk

A FIRST APPROACH TO NANOTOXICOLGOY

1. *In vitro* tests of NPs taking into account:
    1. Size
    2. Chemical nature
    3. Coating

2. To evaluate the possible:
    1. Cytotoxicity
    2. Genotoxicity
    3. Embryotoxicity
    4. Internalization

EMBRYOTOXICITY

Embryotoxicity of cobalt ferrite and gold cobalt nanoparticles: A first in vitro approach

Claudia Di Guglielmo, David Ramos López, Joaquín De Lapuente, Joan Maria Llobet Mallafre, Miquel Borràs Suàrez.

Reproductive Toxicology 30 (2010) 271-276.

	Cytotoxicity assay: MTT test		Embryonic Stem Cell Test (EST)	
	IC_{50} 3T3	IC_{50} D3	ID_{50} D3	Classification
17 ± 3 nm cobalt ferrite NP covered with gold, aminosilanes and thiol-silanes NP	3518.75	3585.35	1913	Non-embryotoxic
12 nm gold NPs coated with 5kDa MW Hyaluronan	2931.85	852.74	166.38	Weakly embryotoxic
17 ± 3 nm cobalt ferrite NP covered with aminosilanes and thiol-silanes	680.95	276.02	39.45	Weakly embryotoxic
$CoFe_2O_4$	243.91	20.05	36.86	Weakly embryotoxic
$HAuCl_4 \cdot 3H_2O$	7.69	7.7	4.96	Weakly embryotoxic
5-FU	0.20	0.10	0.03	Strong embryotoxic
5-FU control	0.20	0.10	0.02	Strong embryotoxic

Weakly embryotoxic:

gold salt > cobalt ferrite salt > cobalt ferrite NPs coated with silanes > gold nanoparticles coated with hyaluronic acid.

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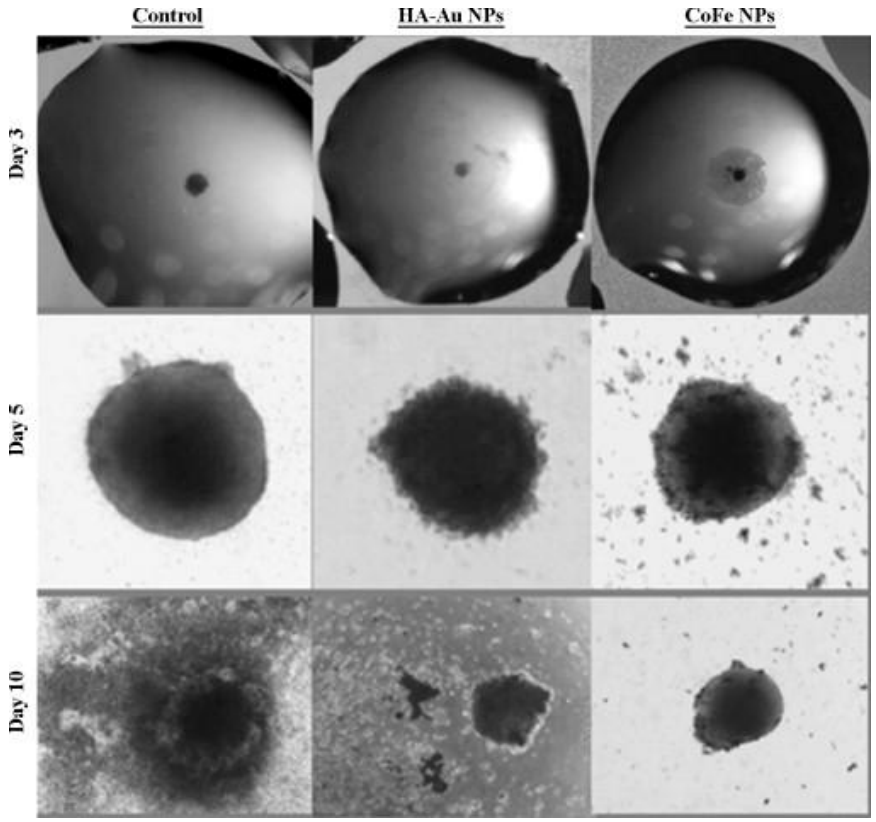
Standard protocol for EST: unsuccessful with NPs.



NPs allow the bodies to be formed.

NPs are laid on the bodies.

Embryonic bodies exposed to NPs do not adhere after day 5 and do not extend properly to the surface of the plate

The shape of the bodies appears irregular and the diameter is smaller if compared to the controls.

- We addressed the suitability of exposing the cells to the toxic agent only for 5 days.
- Confirmation of the validity of this solution:
  - A parallel test with the positive control 5-FU gave confirmation:
    - The ID_{50} concentrations obtained were equivalent.

IN VITRO GENOTOXICITY AND CHRONIC CYTOTOXICITY

Chronic cytotoxicity

- MTT assay
- BALB/c 3T3 cells
- 10 days on exposure to:

	IC_{50}
Gold salts	7.67 µg/mL
12 nm gold NPs coated with 5kDa MW Hyaluronan	3935.81 µg/mL
12 nm gold NPs	1349.77 µg/mL

Genotoxicity

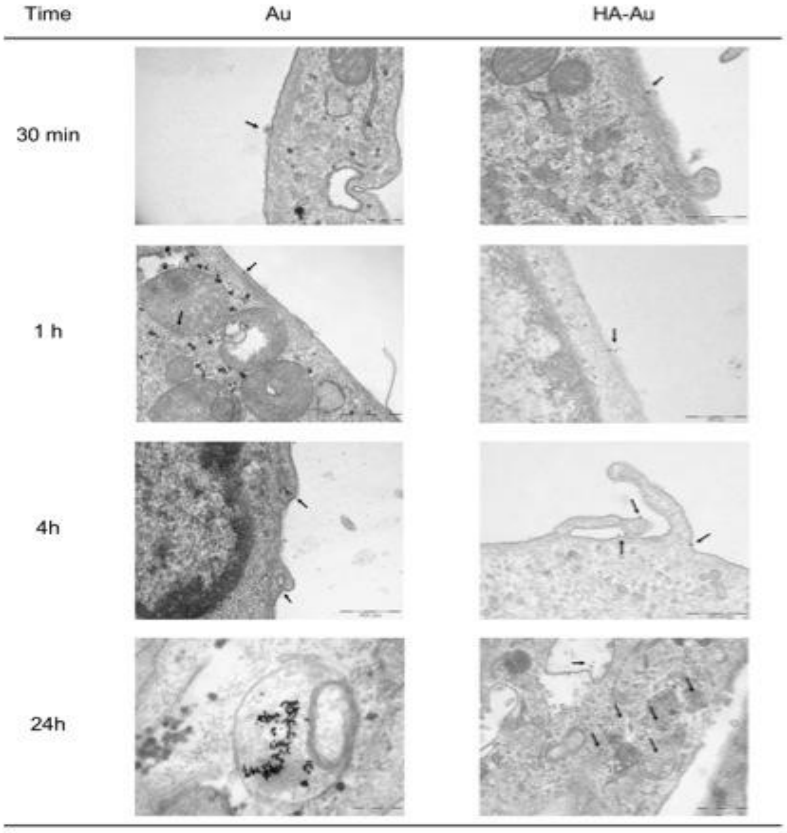
- Comet assay
- BALB/c 3T3 cells
- Exposure
  - To a subtoxic concentration of:
    - 500 µg/mL for NPs
    - 5 µg/mL for gold salts

	DNA damage		
	Gold salts	Coated gold NPs	12 nm gold NPs
15 min			↑
30 min			↑
4 h			↑↑
24 h		↑	↑↑
48 h	↑	↑	↑↑

IN VITRO CELL INTERNALIZATION

Transmission Electron Microscopy (TEM)

- BALB/c 3T3 cells
- Sub-cytotoxic dose of 500 µg/mL of NPs



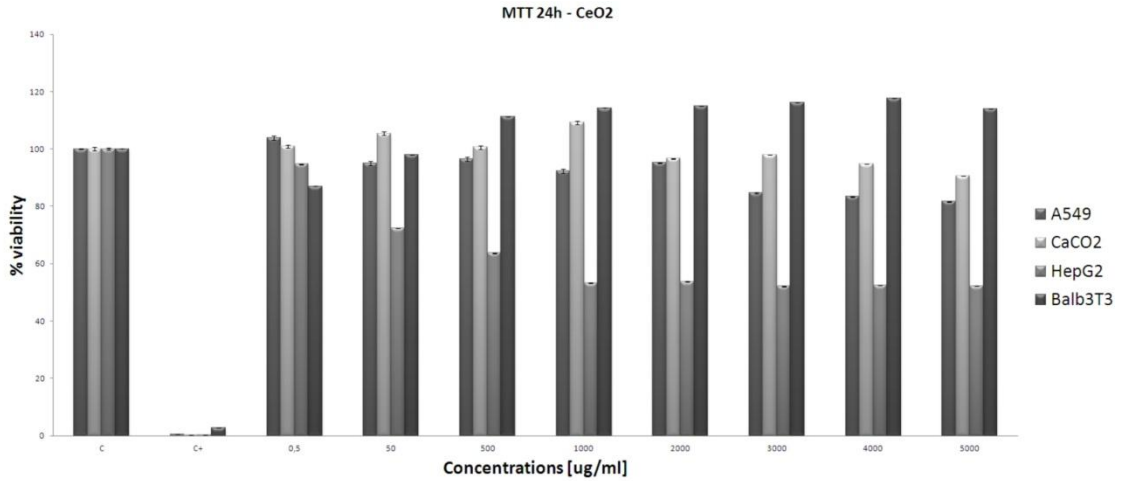
Confocal microscopy

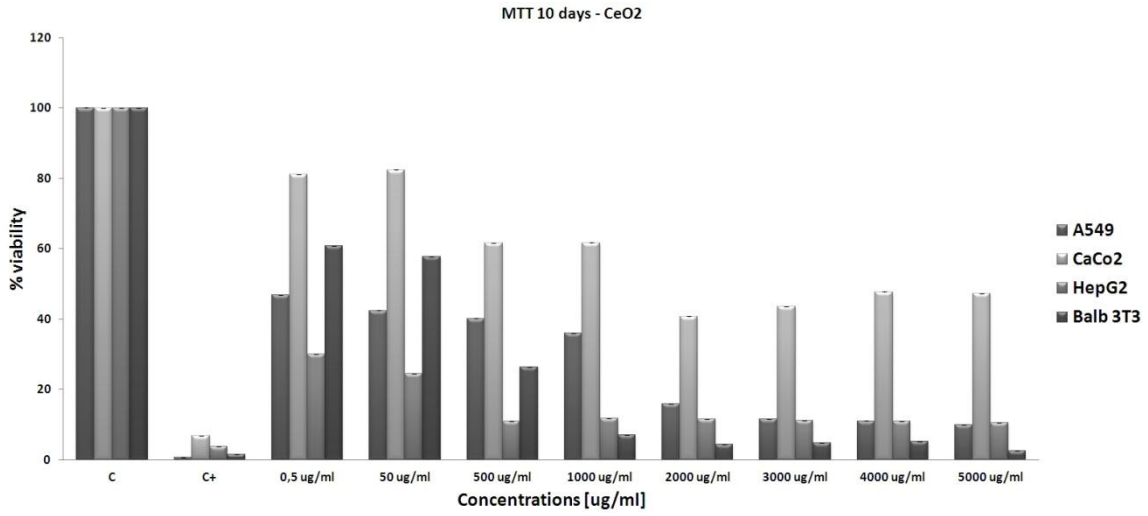
- BALB/c 3T3 cells
- Sub-cytotoxic dose of 500 µg/mL of NPs
- Exposure for:
  - 24 h
  - 4 h
  - 1 h
  - 30 min

	Endosomes	Lysosomes
HA-AuNPs	↑ (up to 24h) → ↓	↑ (↓ with time)
AuNPs	≈	↑ (sp at 4h)

CERIUM OXIDE NPs

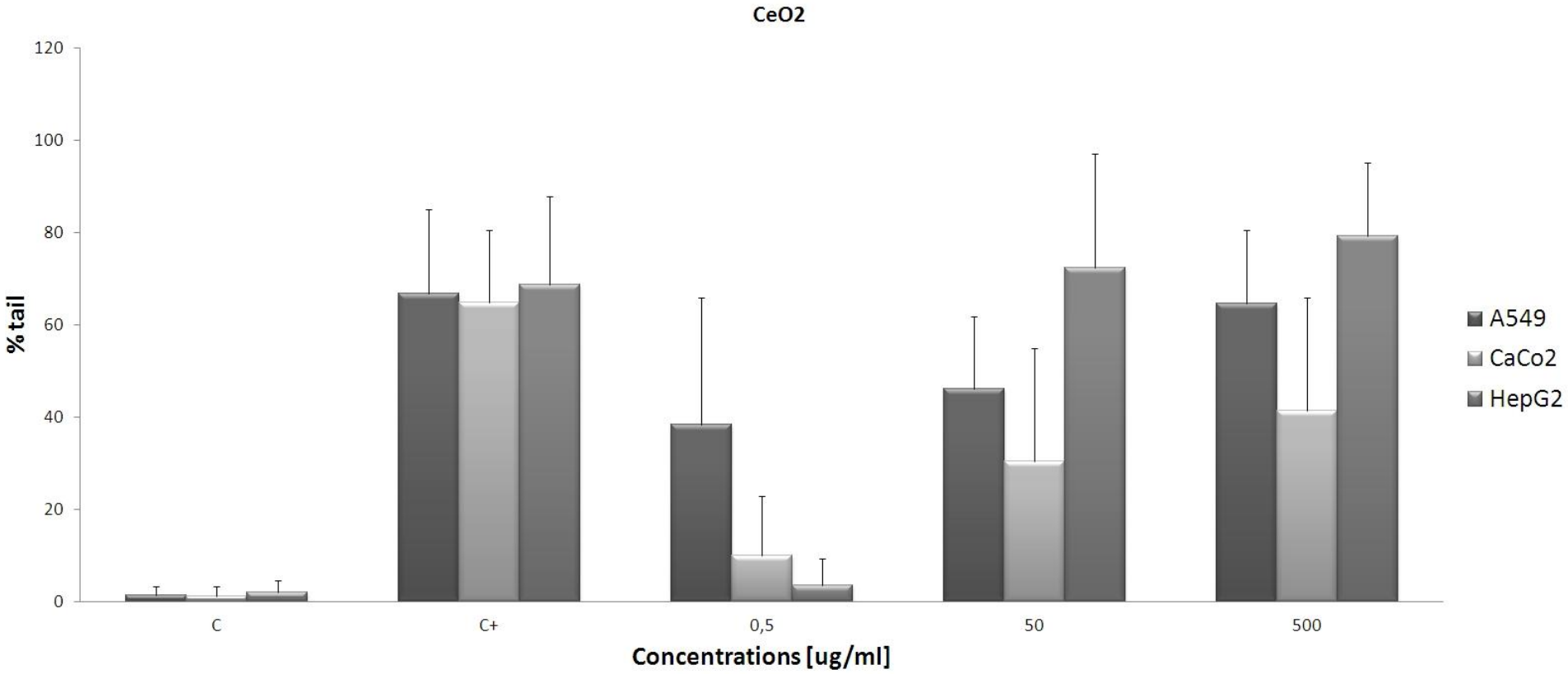
Cytotoxicity: No acute toxic effect





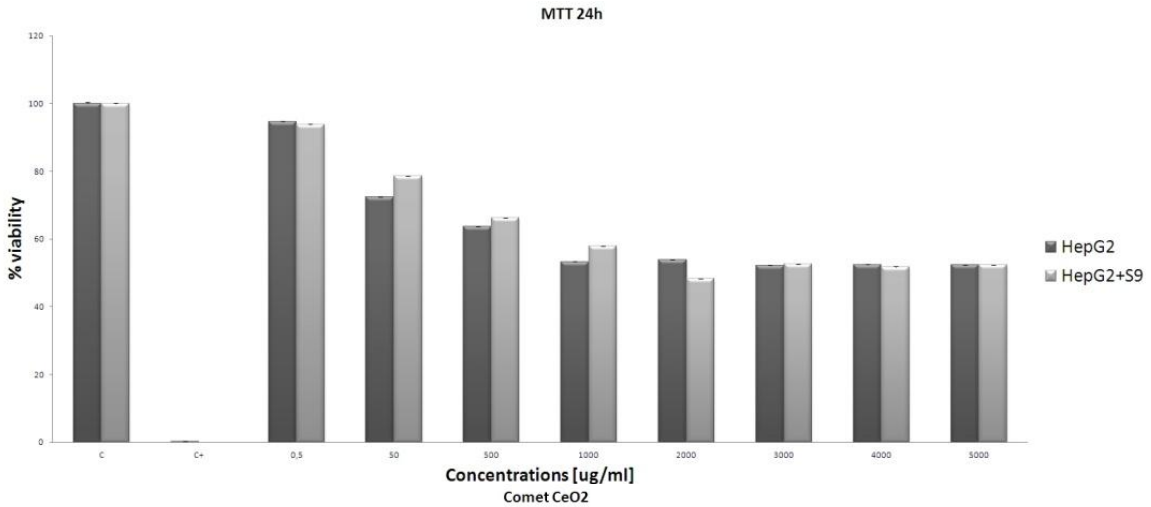
Data reported by Laura di Marzi

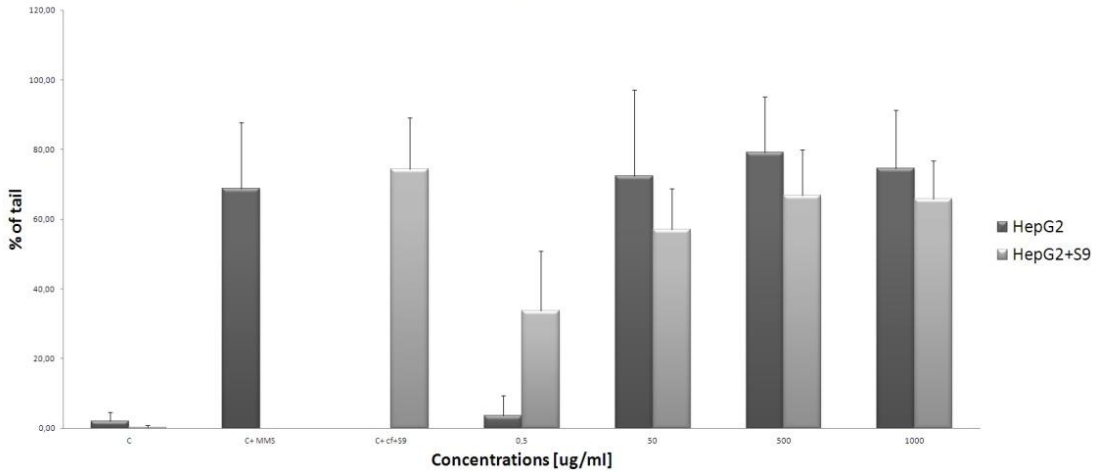
CERIUM OXIDE NPs Genotoxicity: Very high toxic response



Data reported by Laura di Marzi

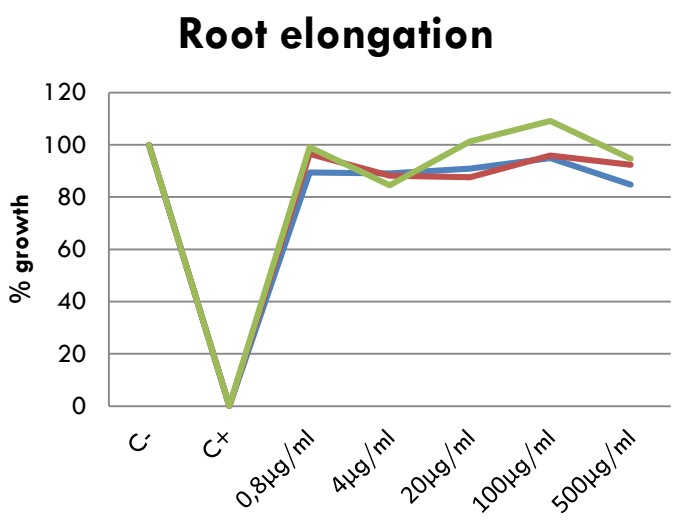
CERIUM OXIDE NPs Effect regarding metabolic activation

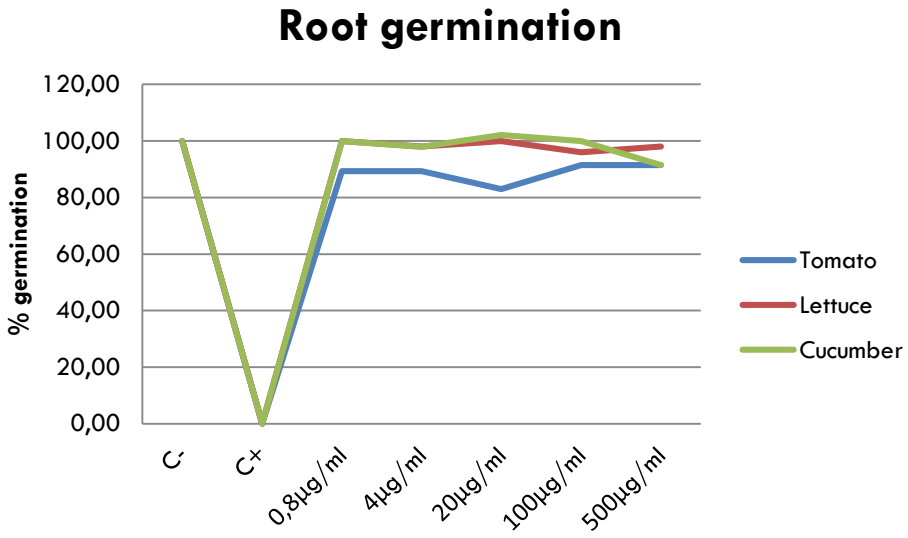




Data reported by Laura di Marzi

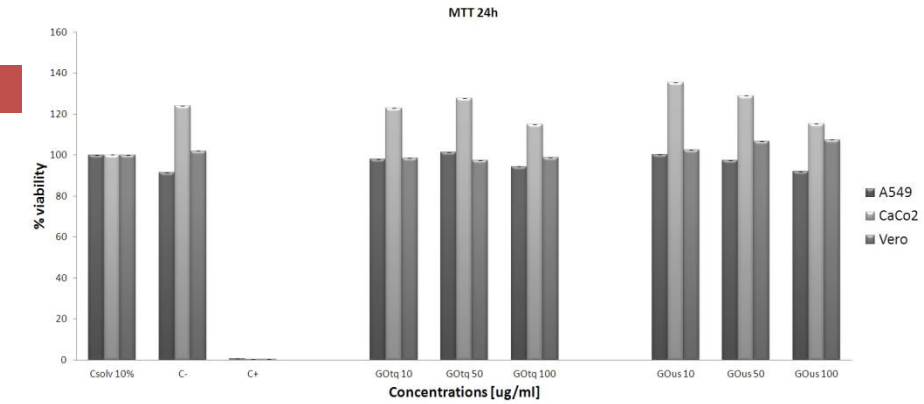
CERIUM OXIDE NPs Ecotoxicity: Seed germination test

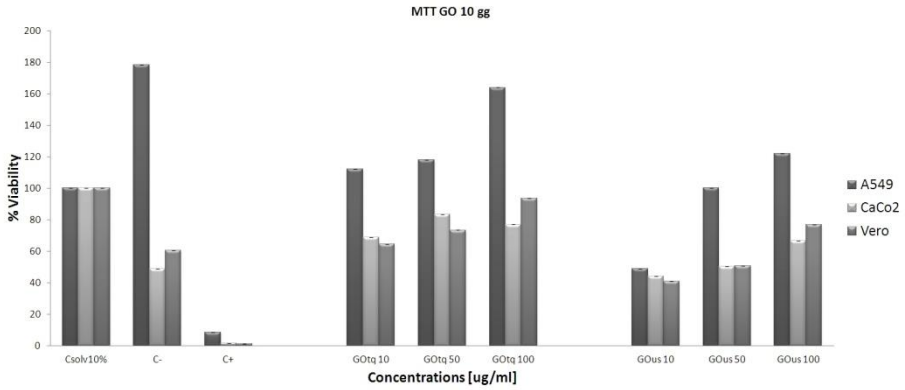


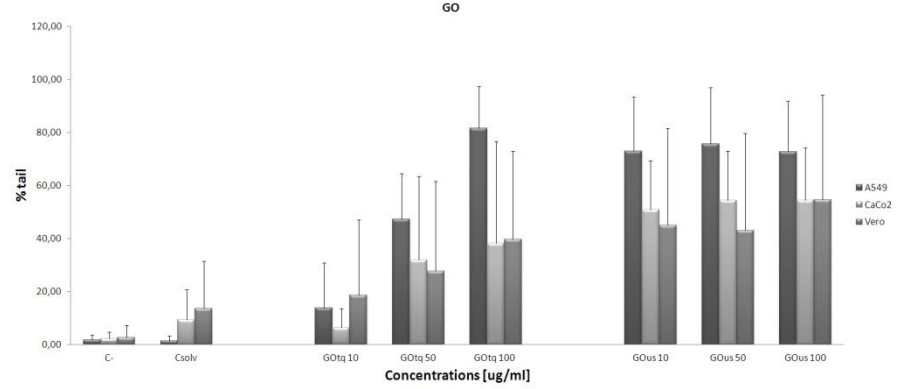


Smaller seeds showed a higher level of toxic effects than the bigger ones at the same concentration of nanoparticles

GRAPHENE OXIDE NPs







There are high differences between exposition periods: Chronic>Subacute>Acute

Genotoxic dose-response depend on the nanoparticle sinthesys

IN VIVO

Inhalation exposure to three types of gold NP for 21 days (*Rattus novergicus*)

Systemic toxicity

- No rat showed severe clinical signs
- All animals gained weight during exposure
- Blood chemistry analysis:
  - all values were within reference values
- Hematology parameters:
  - All values were within reference values
  - No inflammatory response was observed after 3 weeks of exposure, in any group of treatment.
- Leukocytic formula:
  - 100 nm gold NP 7.5 mg/day group:
    - ↑ lymphocytes
    - ↓ granulocytes

Malondyaldehid (MDA) determination

Table IV: MDA results

Exposure group	Time	Plasma	Lung	Liver
		µmols MDA/L plasma	nmols MDA/g lung	nmols MDA/g liver
		Mean ± S.D.	Mean ± S.D.	Mean ± S.D.
12AuNP 7,5 mg/day	Initial	6,601 ± 2,47	-	-
	Final	10,030 ± 2,51	157,553 ± 30,32	283,534 ± 51,99
12AuNP 3,75 mg/day	Initial	5,923 ± 1,30	-	-
	Final	6,741 ± 2,84	189,770 ± 21,78	198,815 ± 74,36
12AuNP 1,875 mg/day	Initial	7,339 ± 1,50	-	-
	Final	6,381 ± 1,90	191,539 ± 35,23	236,898 ± 88,60
HAAuNP 7,5 mg/day	Initial	5,247 ± 0,80	-	-
	Final	9,889 ± 2,42	194,554 ± 53,976	230,384± 59,05
HAAuNP 3,75 mg/day	Initial	5,633 ± 0,89	-	-
	Final	9,737 ± 2,32	191,993 ± 56,73	231,252 ± 58,43
HAAuNP 1,875 mg/day	Initial	8,618 ± 0,79	-	-
	Final	7,056 ± 1,88	184,563 ± 41,54	211,239 ± 36,22
100AuNP 7,5 mg/day	Initial	5,683 ± 1,50	-	-
	Final	9,659 ± 3,06	188,879 ± 33,98	272,639 ± 46,10
100AuNP 3,75 mg/day	Initial	8,216 ± 1,84	-	-
	Final	6,974 ± 1,50	185,576 ±49,63	240,176 ± 71,57
100AuNP 1,875 mg/day	Initial	7,372 ± 0,35	-	-
	Final	8,273 ± 2,24	205,737 ± 53,02	222,498 ± 72,19
Control	Initial	6,268 ± 0,40	-	-
	Final	7,800 ± 2,33	182,050 ± 24,75	232,406 ± 57,02

IN VIVO

Inhalation exposure to three types of gold NP for 21 days (*Rattus novergicus*)

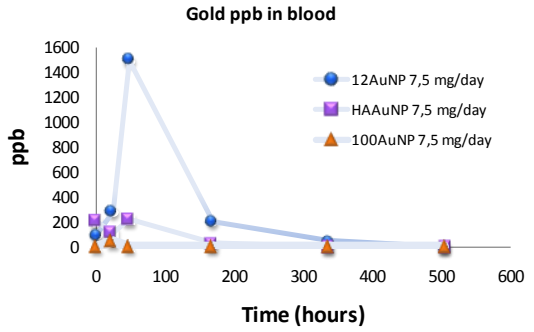
Comet assay

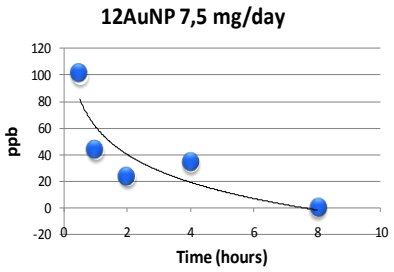
Table VI: % tail intensity	
Exposure group	Mean ± S.D.
12AuNP 7,5 mg/day	27,51 ± 11,99
12AuNP 3,75 mg/day	8,379 ± 8,16
12AuNP 1,875 mg/day	14,390 ± 11,62
HAAuNP 7,5 mg/day	13,361 ± 12,78
HAAuNP 3,75 mg/day	8,261 ± 8,64
HAAuNP 1,875 mg/day	9,041 ± 10,33
100AuNP 7,5 mg/day	20,112 ± 12,97
100AuNP 3,75 mg/day	16,142 ± 15,09
100AuNP 1,875 mg/day	10,890 ± 10,37
Control	12,568 ± 9,97

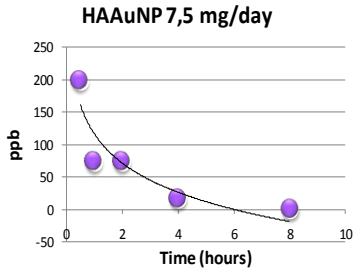
- Increased percentage of DNA in tail in high dose groups of 12AuNP and 100AuNP
- Dose-dependent for 100 AuNP treatment

Tissue gold content determination

	Table VII: ppb of gold in tissue								
Exposure group	Testicle	Olfactory bulb	Lung	Liver	Lymph node	Spleen	kidney	Pancreas	Ovary
12AuNP 7,5 mg/day	<15	<52	49501	<9	<62	<20	<44	<53	<17
HAAuNP 7,5 mg/day	<12	<43	33894	<13	<46	<18	<61	<27	<38
100AuNP 7,5 mg/day	<9	<28	1806,1	<14	<35	<14	<28	<32	<23

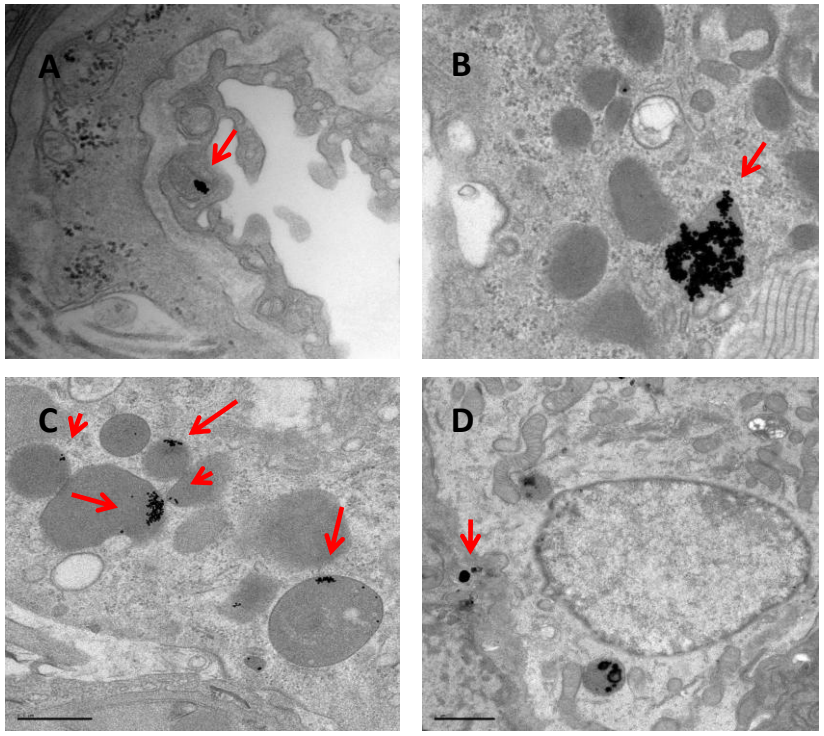






IN VIVO

Inhalation exposure to three types of gold NP for 21 days (*Rattus novergicus*)



- Predominantly found in
  - macrophage cells in the lung
  - inside vesicles

CONCLUSIONS (1/2)

- Lack of standard procedure protocols for nanotoxicology.
- The standard tests for chemicals are not always useful for these kinds of materials.
- The great variability between NPs makes difficult to establish a consensus about which methods are really useful to test their toxicity.
- Some of the most important physicochemical properties that must be characterized:
  - Size
  - Solubility
  - Surface area
  - Surface charge
  - Surface composition
  - Agglomeration/Dispersability
  - Shape
  - Crystallinity
  - Chemical composition
- It is necessary:
  - Get consensus about how to classify nanomaterials into categories
  - Use reference materials to compare toxicity results in different assays
  - Reach agreement on a battery of *in vitro* screening tests.

CONCLUSIONS (2/2)

- Taking into account our experience:
  - Gold NPs affect cell growth only at very high concentrations, as IC_{50} values demonstrated.
    - → Gold NPs were considered as non-cytotoxic for 3T3 cells.
  - Cerium oxide and graphene NPs revealed no acute toxic effect, but can produce a decrease in % of viability after a chronic effect.
  - NPs have shown to be less cytotoxic and less embryotoxic than their salt counterparts, and this lesser toxicity is modulated by the kind of biocompatible coating applied.
  - NPs resulted to be genotoxic starting from their internalization times
    - Uncoated gold nanoparticles, which are internalized more quickly and efficiently, cause DNA damage after 4 hours of incubation
    - Gold NPs coated with hyaluronan induce damage later in time, after 24 hours
    - Cerium oxide NPs alterate the DNA structure in the *in vitro* tests inducing genotoxic effects.
    - Graphene Oxide NPs obtained from different synthesys showed different genotoxic responses.
    - DNA damage may be originate from indirect mechanisms
  - Lysosomes levels rised following time, indicating that cells prepared themselves to hold nanoparticles.
  - Cerium oxide NPs in the presence of a well known oxidant compound are able to protect the cells from oxidative stress damages due to its chemical nature.
  - Chronic *in vivo* study after inhalation exposure showed:
    - No systemic toxicity
    - Increase in the number of lymphocytes for 100 nm gold NPs
    - Oxidative stress was detected in animals treated with higher doses of gold NPs.
    - 100 NPs showed a dose-response effect regarding genotoxicity
    - All gold NPs were able to cross the respiratory barrier, reflecting different patterns of distribution regarding size and coating:
      - Coating is improving the distribution of NPs
      - Smaller gold NPs cross easier into the blood
      - Gold NPs were only detected in the lung, mainly located in macrophages within cellular vesicles and rarely in the cytoplasm

Thank you

Please, feel free to contact me: cporredon@pcb.ub.cat