Usefulness of currently available *in vitro* methods for toxicity assessment of nanoparticles and nanomaterials

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SCOPE OF THE LECTURE

- **Basic definitions and issues**
  - **Why should we care about health effects of a substance in nanosize?**
  - **The Three Principles of Nanotoxicology**
- **Why in vitro methods?**
- **Advantages and disadvantages of *in vitro* testing**
- **Current in vitro cytotoxicity assays used in nanotoxicology**
- **Nanoparticle properties influencing in vitro toxicity assays**

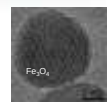
Nanotechnology/nanomaterials

- **Nanostructure/Nanomaterial**: ***Any form of a material that is composed of discrete functional parts, many of which have one or more dimensions of the order of 100 nm or less***

  - ***Nanosheet: A discrete entity which has one dimension of the order of 100nm or less and two long dimensions***

  - ***Nanorod/nanofibre/nanotube):A discrete entity that has two dimensions of the order of 100 nm or less***

  - ***Nanoparticle: A discrete entity that has three dimensions of the order of 100 nm or less***



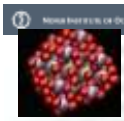
Why the material in nanoscale may differ from bulk chemical ?

There is no intrinsic risk associated with nanoscale per se.

However:

Nanoparticles are unique:

- since between 1 and 100 nm the physical behaviour of particles changes from classical physics to quantum physics with decreasing particle size.
- Due to high energetic adhesive forces close to the surface, the particles are either agglomerated to their neighbours, glued to the next available surface or work like an activated charcoal filter towards other small molecules.



particle size and number of surface expressed molecules

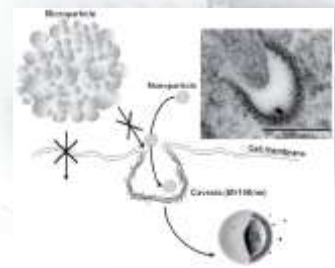
	Total number of molecules in nanoparticle	% of atoms on a surface
	13	92
	55	76
	147	63
	309	52
	561	45
	1415	35

- Unique physico-chemical properties in nanoform
- High biological activity
- Fast growing production volume of chemicals in nanoform and their new applications
- Difficult to control in the environment, both occupational and general

The Three Principles of Nanotoxicology

Three Principles

I. Nanosized objects can enter cells much more easily, faster and in higher concentrations compared with bigger particles or ions as they are able to utilize a whole set of uptake pathways

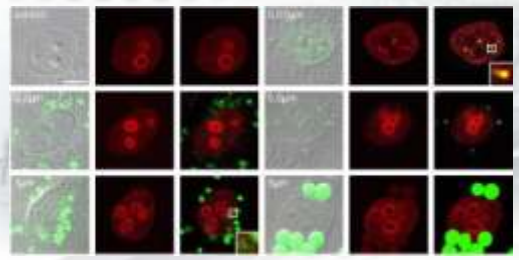


- ZnO nanoparticle (10–50 nm) contains 50.000 to 8 million Zn atoms
- With a typical cell volume of ~500 fL this quantity of atoms, evenly distributed in the cell, would correspond to Zn concentration of 150 nM to 25 µM
- Concentrations above 100 µM of Zn are usually harmful !

Trojan Horse Mechanism

Angew. Chem. Int. Ed. 2011, 50:1260–1278

Nanoparticles may enter the cell nucleus!



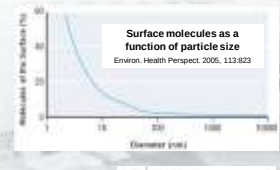
Silica NPs (0.07 µm) enter the cell nucleus where they induce aberrant clusters of topoisomerase I in the nucleoplasm and form intranuclear protein aggregates leading to inhibition of replication, transcription, and cell proliferation

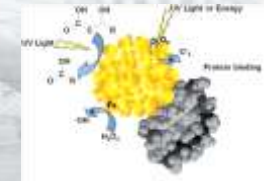
human lung (A549) and epithelial (HEp-2), rat lung epithelial (RLE-6TN), and murine neuronal (N2a) cells

Exp Cell Research 2005, 305:51–62

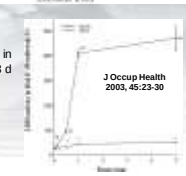
Three Principles

II. The surface principle - smaller particles have an increased surface to-volume ratio





- LDH activity in BALF from rats at 3 d after instillation of:
  - Std-Ni (5 µm)
  - Uf-Ni (20 nm)

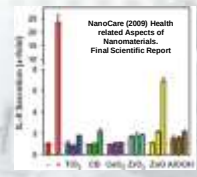


- Crystalline structures or quantum effects provoke energy absorption and transfer, leading to formation of ROS or the degradation of hydrocarbons
- Nanoparticles bind to biological macromolecules of comparable size as proteins or DNA

Angew. Chem. Int. Ed. 2011, 50:1260–1278

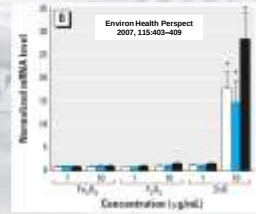
Three Principles

III. The principle of material - different material compositions exhibit different properties and react in different ways with cells or biomolecules



A549 cells exposed to:

- TiO_2 10–20 nm
- carbon black (CB) 15 nm
- CeO_2 20 nm
- ZrO_2 10-25 nm
- ZnO 40 nm
- AlOOH 40 nm



mRNA levels of ICAM-1, IL-8, and MCP-1 in human aortic endothelial cells incubated with nanoparticles for 2 hr:

- Fe_2O_3 - two size modes: 47 nm and 5 nm
- Y_2O_3 - 20–60 nm
- ZnO - 100–200 nm X 20–70 nm

Why in vitro?

Advantages and disadvantages of *in vitro* testing

Why in vitro?

Advantages:
- **Ethical aspects** (3R principle and the need to reduce animal testing)
- **Relative speed** (High-Throughput Screening possibility)
- **Relative lower costs** in comparison to *in vivo* testing
- **Easy in manipulating the toxicity mechanisms**

Disadvantages:
- *In vitro* systems **lack the complexity of animal models** or the human body
- The **metabolic activity** of standardized cell lines has often **not** been **comprehensively characterized**
- *In vitro* systems have **no value for the prediction of biodistribution and target organ** toxicity for the applied chemical and its metabolites
- **A dose–response** relationship can probably only be reliably determined *in vivo*

REACH

(*R*egistration, *E*valuation, *A*uthorisation of *Ch*emicals)

All substances, both already existing (i.e. brought onto the EU market before 1981), and new (i.e. brought onto the market after 1981), produced or imported in quantity exceeding ≥1 tonne, and for which there is no sufficient toxicological documentation **should be tested** for safety for human health and environment

In force since 1.06.2007

REACH: 1200 pages, 1/3 concerns testing, mainly on animals

ALTERNATIVE TESTING
„A scientific objective for the European Union is the development and validation of alternative techniques"...„

Available *in vitro* culture models

in vitro culture models

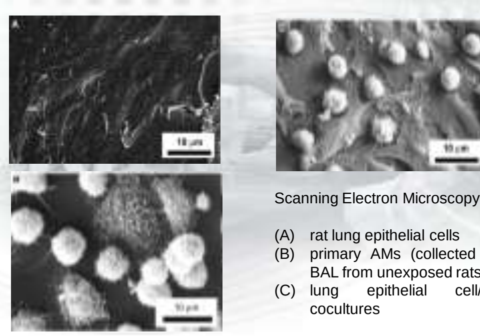
Models of mono-cultures

Monocyte-derived macrophages	(Waldmann et al. 2007)
Monocyte-derived dendritic cells	(Blank et al. 2007; Rothen-Rutishauser et al. 2007, 2005)
Cell lines	
Airway epithelial cells	
Calu-3	([illegible] et al. 2004; [illegible] et al. 2007; [illegible] et al. 2006)
16HBE14o-	([illegible] et al. 2008; [illegible] et al. 2006)
BEAS-2B	(Herzog et al. 2007; Jang et al. 2006; Park et al. 2007; Veranth et al. 2007)
Alveolar epithelial cells	
A549	(Duffin et al. 2007; Park et al. 2007; Stearns et al. 2001)
Immortalized human alveolar type 2 cells with alveolar type 1 phenotype	(Kemp et al. 2008)
Macrophages	
THP-1	(Chen et al. 2006; [illegible] et al. 2008; [illegible] et al. 2008; Wottrich et al. 2004)
Fibroblasts	
MRC-9	(Limbach et al. 2005)
Mesothelial cells	
MSTO-211H	(Kaiser et al. 2006; Wick et al. 2007)

Arch Toxicol DOI 10.1007/s00204-010-0560-6

in vitro culture models

Models of bi-cultures

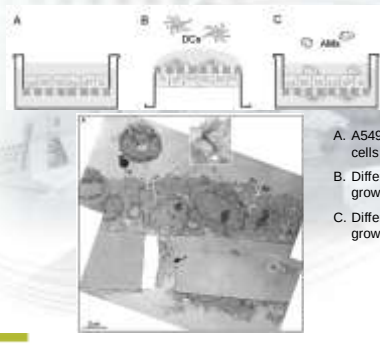


Scanning Electron Microscopy of:

(A) rat lung epithelial cells
(B) primary AMs (collected via BAL from unexposed rats)
(C) lung epithelial cell/AM cocultures

Toxicol. Sci. 2007, 97:163-180

Triple-cell models of respiratory system



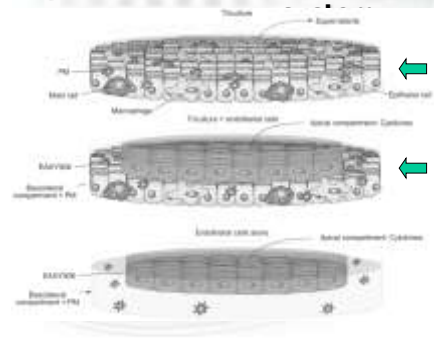
A. A549 (*carcinoma bronchoalveolare*) cells growing on top side of the filter

B. Differentiated dendritic cells (DC) growing on basal side of the filter

C. Differentiated macrophages (AM) growing on top of A549 cells

Am J Respir Cell Mol Biol. 2005, 32: 281–289

Quadruple-cell models of respiratory tract



Tricultures
A549 + THP-1 + HMC-1 in a 10:2:1 ratio

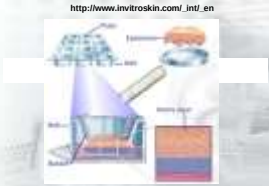
EAHY926 cells were introduced in inserts above the tricultures

Eur Respir J 2008; 32:1184–1194

3D human reconstructed skin models

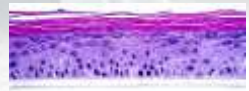
EPISKIN™ EpiDerm™ SkinEthic™ EST-1000

http://www.invitroskin.com/_int/_en



in vivo

RHE



in vitro

Composed of human **keratinocytes** and human dermal **fibroblasts** within a collagen gel, the epidermis is **fully differentiated**, expressing a range of differentiation markers and containing lipid lamellar in its cornified layer

In vitro assays for assessing the pathogenic potential of nanomaterials

Endpoints	
Cytotoxicity:	Trypan blue exclusion assay, tetrazolium reduction assays, clonogenic (CFE) assay, LDH assay, TUNEL assay, Apostain technique, flow cytometry with PI, 7AAD, and/or annexin V, lipid peroxidation, cytochrome c release from mitochondria, caspase activation
Proliferation:	DNA content, [^{3}H]thymidine incorporation, BrdU incorporation, Ki-67, and detection of PCNA
Genotoxicity:	Ames assay (*S. typhimurium* or *E. coli*), detection of DNA base modifications, karyotype analyses (induction of chromosome aberrations and micronuclei), comet assay
Gene expression:	Northern blot analyses, ribonuclease protein assays (RPA), real-time PCR, PCR assays, microarrays

Wiley Interdiscip Rev Nanomed Nanobiotechnol 2010, 2: 219–231

Current in vitro cytotoxicity assays used in nanotoxicology

Cell viability - the most commonly investigated parameter in cytotoxicity testing; different endpoints are currently utilized to assess the actual state of cultured cells *in vitro*

- Detection of mitochondrial activity (colorimetric MTT, MTS, XTT, WST-1 reduction assays)
- ATP content of cells
- Reduced glutathione levels
- Detection of intact lysosomes via neutral red uptake
- Trypan blue exclusion test and detection of LDH release upon necrosis
- Detection of apoptosis: TUNEL and Annexin V/propidium iodide staining, Caspase-3

Current in vitro cytotoxicity assays used in nanotoxicology

Stress response

- Detection of reactive oxygen species (H2DCF-DA, 2',7'-dichlorodihydrofluoresc(e)in diacetate)
- The ratio of reduced glutathione (GSH) versus its oxidized form (GSSG)
- Free radical formation through the colorimeter thiobarbituric acid method and more specific by spin trapping agents and electron spin resonance measurements of the stable adducts formed
- Adduct formation of hydroxyl radicals with 8-OH-deoxyguanosine
- Inflammatory response (*in vitro* studies of inflammatory marker production via enzyme linked immunosorbent assay (ELISA): IL-8, TNF-, IL-6, IL-1b, MIP-2, etc.)

Nanomaterial properties influencing *in vitro* toxicity assays

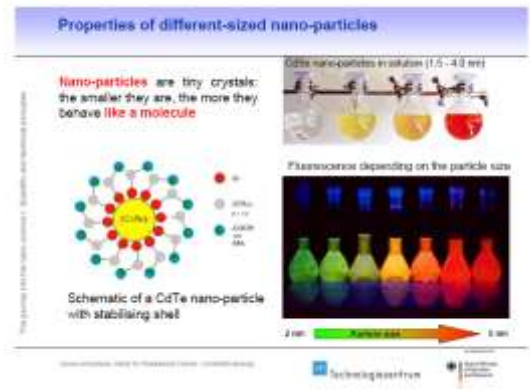
influence on assays

High adsorption capacity

- due to their large surface per unit mass, NPs display an increased adsorption capacity and biological reactivity as compared to the bulk material
  - indirect effects through the adsorption of nutrients and growth factors from culture media
  - direct influence on the assay outcome when protein concentration or activity is utilized to evaluate particle toxicity
  - direct interaction with other assay components (e.g., substrates, dyes)
  - high adsorptive particles could potentially bind contaminating compounds during the production process

Nanoparticle interference with cytotoxicity assays

Kroll et al., Eur. J. Pharm. Biopharm. 2008



influence on assays

Catalytic activity - high surface/mass relationship of nanosized materials results in an excess surface energy enhancing catalytic activity
  - metal oxide and silica NPs, fullerenes were shown to produce ROS in cell-free systems
  - ROS production was 100–1000 times faster with 2–4 nm-sized ZnO NPs (4-5 nm) than with 100 nm-sized particles (Environ Sci Technol. 1994, 28:776–785)
  - photoactivated TiO_2 and ZnO NPs were shown to degrade anionic dyes like erythrosine, while SWCNTs may interfere with MTT viability tests by reducing the substrate MTT

influence on assays

Acidity/alkalinity - the most widely used *in vitro* assays are pH-dependent, and may thus be influenced by acidic or basic nanoparticles if these remain in considerable amounts in the assay mixture

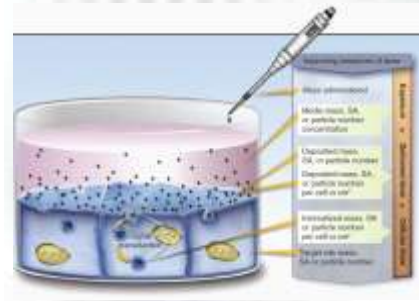
Magnetic properties - some metal oxide NPs (e.g. Fe_2O_3) are superparamagnetic and generate strong, local magnetic fields which lead to the production of free radicals that in turn may interfere with cytotoxicity methods based on redox reactions

Dissolution - NPs designed to dissolve in aqueous solutions (e.g. some QD) or showing an intrinsic dissolution in aqueous media (e.g. ZnO), will release metal ions or trace metals when introduced into biological media

Eur J Pharmaceutics Biopharmaceutics 2009, 72:370–377

Thank you for your attention

Defining dose for nanoparticles *in vitro*



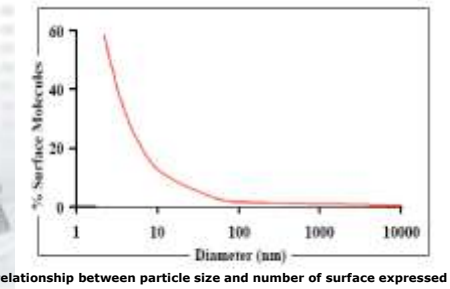
Different dose metrics:

µg/ml
µg/cm² culture dish
µg/10⁶ cells
particle number/10⁶ cells
particle number/cm²

Nanomedicine 2011, 6(5)

- Well-validated positive and negative controls („gold standard nanoparticles") urgently needed!

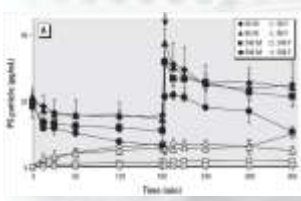
Dalian 33



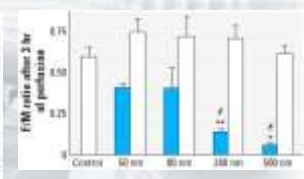
Inverse relationship between particle size and number of surface expressed molecules.

In the size range <100nm, the number of surface molecules (expressed as a % of the molecules in the particle) in inversely related to the particle size. For instance, in a particle of 30 nm size, about 10% of its molecule are expressed on the surface, whereas at 10 and 3 nm size the ratio increase to 20% and 50%, respectively. Because the number of atoms or molecules on the surface of the particle determine the material reactivity, this is a key to defyining the biological properties of nanoparticles (Nel at al., Science 311:622-627, 2006, adopted from Oberdörster et al., Env. Hlth Perspect. 113:823- 839, 2005)

Ex vivo systems using whole functional organs



Fetal circuit: increased levels of Polystyrene beads (PS) were measured in the **placental tissue** after a few minutes of perfusion
A second addition of particles after 180 min (arrow) did not further increase the amount of beads in the fetal circuit



Size-dependent barrier capacity of the **placental tissue**. The ratios between fetal (F) and maternal (M) concentrations of ^{14}C-antipyrine (open bars) and Polystyrene beads (PS) (blue bars) were calculated after 180 min of perfusion

Environ Health Perspect 2010, 118:432–436

Impact of nanomaterials tested on genotoxicity test results

- The timing of the tests may have to be adjusted to allow nanomaterial to get access to the nucleus during mitosis. In the in vitro chromosome abberation test, it may be necessary to examine the secong post-treatment metaphase in addition to the first one.
- In the cytokinesis-block micronucleus test in vitro, the exposure could also occur for one cell cycle without cytochalasine B, followed by another cycle in the presence of cyt.B, to examine the cells after 2nd post-treatment mitosis.

Impact of nanomaterials tested on genotoxicity test results

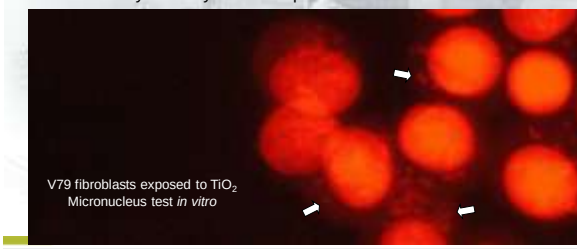
- Nanoparticles that readily pass cellular membranes and may reach nucleus (e.g. SWCN)

  - penetration through bacterial wall different - Ames test, SOS chromotest, other bacterial genotoxicity assays not useful ?
- Direct reactions with DNA and mitotic spindle components (microtubules, kinetochores, centrioles, etc)
- Indirect effects related to potential oxidative stress and inflammatory activity
- Nanoparticles can interact with S9 mix

influence on assays

Optical properties - many nanoparticles display optical properties potentially interfering with the detection system

- due to light-absorptive features, some NPs (e.g. sodium titanate) directly influence the readout in cell viability assays
- NPs used for medical imaging (QD or nanoshells) can absorb and emit light of different wavelengths, and might distort the signal intensity in assays with an optical readout



V79 fibroblasts exposed to TiO_2
Micronucleus test *in vitro*