Available *In Vitro* Methods for Nanotoxicology

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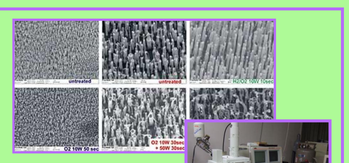
Introduction

Recent innovations in science and technology have allowed the development of human-relevant, predictive, non-animal assays for many biological endpoints. Since statutory requirements do not yet exist for nanomaterials testing, there is ample opportunity for scientists to seek out and use the best science available. Many *in vitro* models can out-perform the animal-based toxicity methods that have traditionally been used in the field of toxicology. Animal models are not only inhumane, but lack predictive value with regard to human responses in many cases.

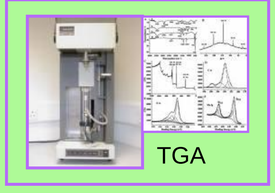
For example, FDA figures show a 92% failure rate for drugs that pass preclinical trials which are based on animal experiments. The field of nanotoxicology can and should avoid this approach and incorporate the most recent science-based technology. This review examines many promising methods currently available to test for nanomaterial safety.

Physical and Chemical Characterization

Complete physical and chemical characterization prior to nanotoxicity testing is imperative. Precise, well-characterized measurements reduce redundancy and allow for standardized analysis. For example the spectroscopic methods listed below can be used to characterize nanomaterials before any toxicity testing is done. Incomplete characterization and the use of non-standardized methods leads to inconsistent and unreliable toxicity data that wastes animals' lives.



SEM/EDX



TGA

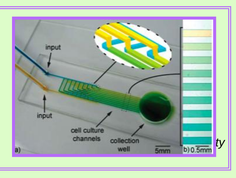
SEM/ EDX → **TEM** → **Raman** → **UV-Vis-NIR** → **TGA**

SEM/EDX, scanning electron, energy-dispersive X-ray spectroscopy; TEM, transmission electron microscopy; TGA, total gamma-absorption spectroscopy

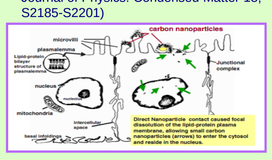
Oxidative Stress & Cytotoxicty in Cells Relevant to Portal of Entry

Several methods exist for assessing activity of nanomaterials using cell types relevant to portal of entry. Cell-type specific assays exist that can measure cytotoxicity as well as gene expression, genotoxicity, or embryotoxicity.

A linear dilution microfluidic device for cytotoxicity assays has been developed. Nine linear dilutions can be tested in parallel on human cells. From Walker *et al.* 2006. Lab Chip 2; 226-32.



Cells representing portals of entry (Caco-2 colon cells and NCI-292 lung cells) show that dose duration creates increasing cytotoxicity. (Panessa-Warren *et al.* 2006. Journal of Physics: Condensed Matter 18; S2185-S2201)



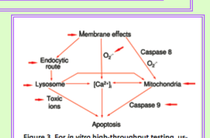


Figure 3. For *in vitro* high-throughput testing, using mechanisms of injury to formulate quantitative structure–activity relationships that incorporate hazardous nanomaterial properties, it is necessary to consider using cellular responses that are shared by a number of toxicological pathways. For instance, if the screening is developed to detect cytotoxicity, one might consider apoptosis as a final common pathway that integrates oxygen radical generation, $[Ca^{2+}]_i$ release, caspase activation, lysosomal damage, liberation of toxic metal ions, and mitochondrial perturbation. Each of these are quantifiable responses that are tied into currently known nanoparticle cytotoxic pathways. The arrows indicate some of the initiation points of cellular toxicity by nanoparticles.

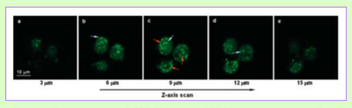
From: Meng, H. et al. *A predictive toxicological assessment of nanomaterials, 2009.*

Additional Relevant References:

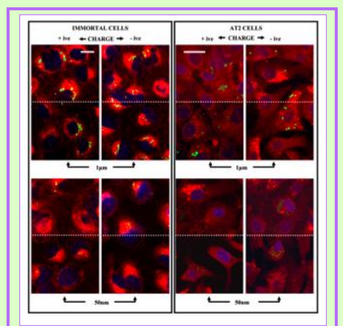
Rogers EJ, et al. *A high throughput in vitro analytical approach to screen for oxidative stress potential exerted by nanomaterials using a biologically relevant matrix: Human blood serum.* Toxicol In Vitro. 2008;
Peters, K., et al. *Effects of nano-scaled particles on endothelial cell function in vitro: Studies on viability, proliferation, and inflammation*. J. Mater, Scie: Mater. Med. 2004; 15: 321-325.
Porter, A.E., et al. *Uptake of C(60) by human monocyte macrophages, its localization and implications for toxicity: studied by high resolution electron microscopy and electron tomography.* Acta Biomater. 2006; 2(4): 409-19.
Ma, S.H., et al., *An endothelial and astrocyte co-culture model of the blood-brain barrier utilizing an ultra-thin, nanofabricated silicon nitride membrane.* Lab Chip, 2005. 5(1): p. 74-85.
Monteiro-Riviere NA, et al. *Multi-walled carbon nanotube interactions with human epidermal keratinocytes.* Toxicol Lett. 2005;155:377–384.
Sayes, C.M., et al., *Correlating Nanoscale Titania Structure with Toxicity: A Cytotoxicity and Inflammatory Response Study with Human Dermal Fibroblasts and Human Lung Epithelial Cells.* Toxicol Sci, 2006.
Zhang XQ, et al. *Effects of nitrovasodilators on the human fetal-placental circulation in vitro. Placenta.* 2001 Apr;22(4):337-46
Bur M, Rothen-Rutishauser et al. *A novel cell compatible impingement system to study in vitro drug absorption from dry powder aerosol formulations.* Eur J Pharm Biopharm. 2008 Aug 17.

Assessing Uptake: Nucleus & Reticulo-endothelial Systems

Concerns about cellular uptake of nanoparticles related to human health as well as environmental safety has prompted many labs to assess the proclivity of nanomaterials to cross cell membranes into endocytic compartments and the nucleus. In order to determine potential risks from nanoparticles, it is critical to determine mode of exposure and assess whether a given nanoparticle will cross cell barriers.



Shown: Translocation of C70-GA across HT-29 cell membranes. From: Salonen, E. et al. *Real-Time Translocation of Fullerene Reveals Cell Contraction.* Small. (2008)



Shown: : Nanoparticles that have traversed AT2 cells membranes. From: Kemp, S. et al *Immortalization of Human Alveolar Epithelial Cells to Investigate Nanoparticle Uptake.* (2008)

Additional Relevant References:

Porter, A.E., et al. *Uptake of C(60) by human monocyte macrophages, its localization and implications for toxicity: studied by high resolution electron microscopy and electron tomography.* Acta Biomater. 2006; 2(4): 409-19.
Hirano S, et al. *Multi-walled carbon nanotubes injure the plasma membrane of macrophages.* Toxicol Appl Pharmacol. 2008 Oct 15;232(2):244-51.
des Rieux A, et al. *An improved in vitro model of human intestinal follicle-associated epithelium to study nanoparticle transport by M cells.* Eur J Pharm Sci. 2007 Apr; 30(5): 380-91.
Xu G, et al. Bioconjugated quantum rods as targeted probes for efficient transmigration across an in vitro blood-brain barrier. Bioconjug Chem. 2008 Jun; 19(6): 1179-85.

Screening for Genotoxicity & Protein Damage

Due to their unique properties, many nanomaterials can cross cellular membranes and intercalate within DNA or damage proteins. Once the portal of entry for a specific nanomaterial is determined, cultured cells from these tissues can be assessed for DNA or protein damage. Several human cell-based assays exist that can be used to characterize genotoxic potential of nanomaterials.

In Vitro Methods Useful for Nanomaterials

- Ames test for bacterial mutation (OECD TG 471)
- *In vitro* chromosomal aberration (OECD TG 473)
- Unscheduled DNA synthesis (OECD 482)
- Sister Chromatid Exchange (OECD 479)

Each of these can be a partial or full replacement for *in vivo* methods. Negative results for these assays preclude the use of additional *in vivo* test confirmation.

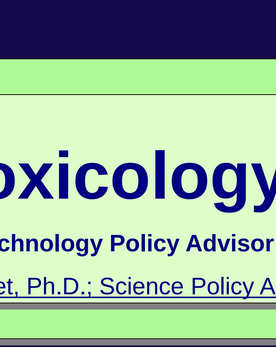


Figure 2. AFM images of pDNA and pDNA−QD conjugates: (A) biotinylated pDNA before QD conjugation, (B) pDNA with ~200 biotin moieties after conjugation with QDs, (C) pDNA with ~20 biotin moieties after conjugation with QDs, and (D) pDNA with ~20 biotin moieties after conjugation with QDs followed by photoactivation at 532 nm for 1 h. The scale bars are (A) 800 nm, (B) 400 nm, (C) 600 nm, and (D) 500 nm.

From: Anas, A. et al. *Photosensitized Breakage and Damage of DNA by CdSe-ZnS Quantum Dots. (2008)*

Assaying plasmid DNA strand breakage when exposed to quantum dots.

Micronucleus Assay

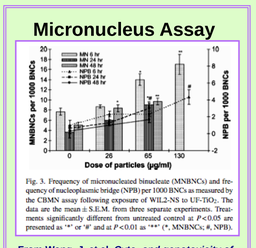


Fig. 3. Frequency of micronucleated binucleate (MNBNCs) and frequency of nucleoplasmic bridge (NPB) per 1000 BNCs as measured by the CBMN assay following exposure of WIL2-NS to UF-TiO_2. The data are the mean ± S.E.M. from three separate experiments. Treatments significantly different from untreated control at $P<0.05$ are presented as '*' or '#' and at $P<0.01$ as '**' (*, MNBNCs; #, NPB).

From Wang, J. et al. *Cyto- and genotoxicity of ultrafine TiO2 particles in cultured human lymphoblastoid cells.* (2007)

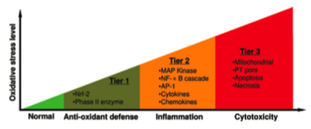
Additional Relevant References:

Kang SJ, et al. *Titanium dioxide nanoparticles trigger p53-mediated damage response in peripheral blood lymphocytes.* Environ Mol Mutagen. 2008 Jun;49(5):399-405.
de Freitas ER, et al. *In vitro biological activities of anionic gamma-Fe2O3 nanoparticles on human melanoma cells.* J Nanosci Nanotechnol. 2008 May;8(5):2385-91.
Colognato R, et al. *Comparative genotoxicity of cobalt nanoparticles and ions on human peripheral leukocytes in vitro.* Mutagenesis. 2008 Sep;23(5):377-82.
Mroz RM, et al. *Nanoparticle carbon black driven DNA damage induces growth arrest and AP-1 and NFkappaB DNA binding in lung epithelial A549 cell line.* J Physiol Pharmacol. 2007 Nov;58 Suppl 5(Pt 2):461-70.

Inflammation and Human Blood Component Effects

Monitoring the inflammatory effects of nanomaterials on *in vitro* human cell types is valuable for the prediction and assessment of the potential toxicity of nanoparticles. Resulting data are analyzed in order to predict a given nanomaterial's proclivity for toxicity and to glean the mechanism of chemical action. The use of human-relevant cell types is of utmost importance as these cell types are comprised of human-specific binding sites and avoid the need for species-to-species extrapolation.

From: Meng H. e t al. ***A predictive toxicological paradigm for the safety assessment of nanomaterials***. *2009.* ACS Nano, 3(7): 16201627.



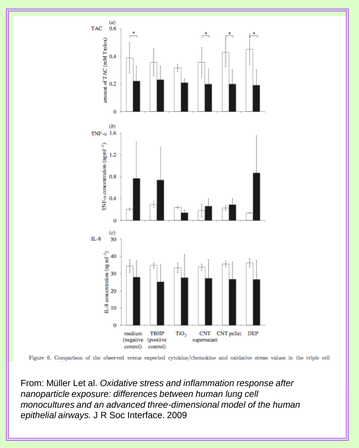


Figure 6. Comparison of the observed versus expected cytokine/chemokine and oxidative stress values in the triple cell

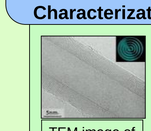
From: Müller Let al. *Oxidative stress and inflammation response after nanoparticle exposure: differences between human lung cell monocultures and an advanced three-dimensional model of the human epithelial airways.* J R Soc Interface. 2009

Additional Relevant References:

Müller Let al. *Oxidative stress and inflammation response after nanoparticle exposure: differences between human lung cell monocultures and an advanced three-dimensional model of the human epithelial airways.* J R Soc Interface. 2009
Meng, H.et al. *A predictive toxicological paradigm for the safety assessment of nanomaterials. 2009.* ACS Nano, 3(7): 16201627.
Choi, SJ et al. *Toxicological effects of inorganic nanoparticles on human lung cancer A549 cells. 2009. J Inorg Biochem 103(3):463-71.*

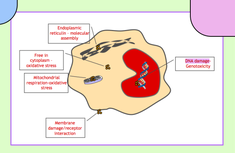
Model for Tiered Nanotoxicity Screening

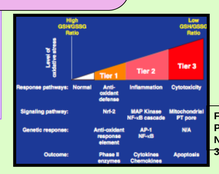
1. **Complete Physical & Chemical Characterization** (SEM, EDX, TEM, TGA, etc.)



TEM image of carbon nanotube

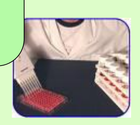
2. ***In vitro* screening tests** (Cytotoxicity, Genotoxicity, Mitochondrial perturbation, DNA damage, etc.)





From Nel *et al.* Toxic Potential of Material at the Nanolevel 2006. Science 311; 622-627

3. **OECD & ECVAM-Approved *In Vitro* Methods** (Pyrogenicity, Phototoxicity, Genotoxicity, Skin penetration, etc.)



Conclusions

Advances in high-tech, analytical methods allow for human-relevant toxicity testing without relying on animals. Electron microscopy and cell culture coupled with diagnostic assays now afford scientists the ability to assess cells/organelles/DNA during toxicological studies and measure the chemical changes in these structures caused by a given nanochemical. The precision with which we are now able to monitor the cell's cyclic changes over time is a more sophisticated diagnostic tool than animal-based toxicological experiments.

It is imperative from a human and environmental safety standpoint that this field be built on a foundation of rigorous and predictive in vitro assays.