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Contemporary Issues in Nanotoxicology: Continuing to Relate Material Properties to Biological Response

Christie Sayes, Ph.D.
Program Manager
Nanotoxicology & Nanopharmacology
Center for Aerosol and Nanomaterials Engineering
RTI International
csayes@rti.org

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Sayes Lab

Department of Vet. Physiology & Pharmacology and the Department of Biomedical Engineering

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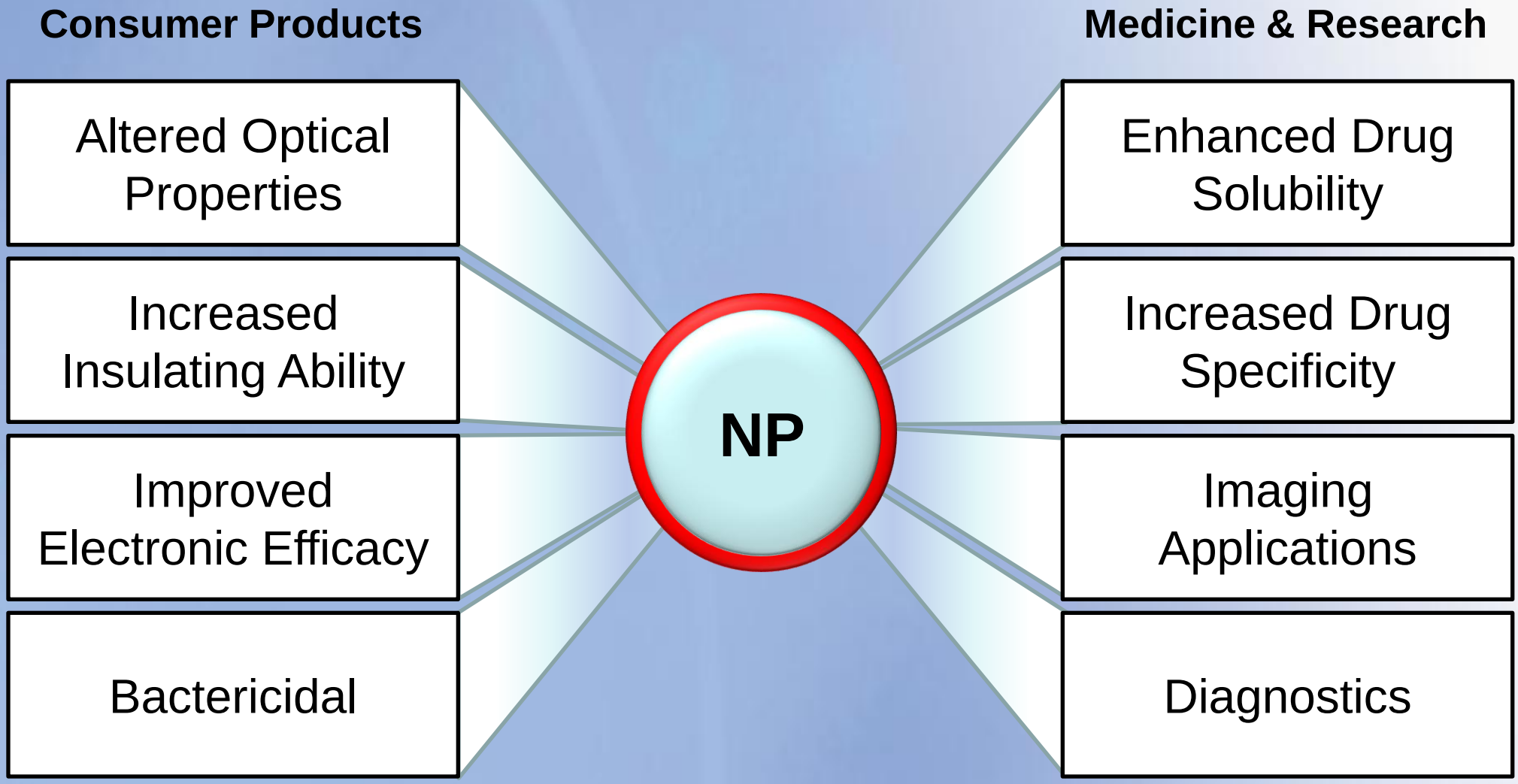
Advantages to Nanotechnology



But what are the risks?

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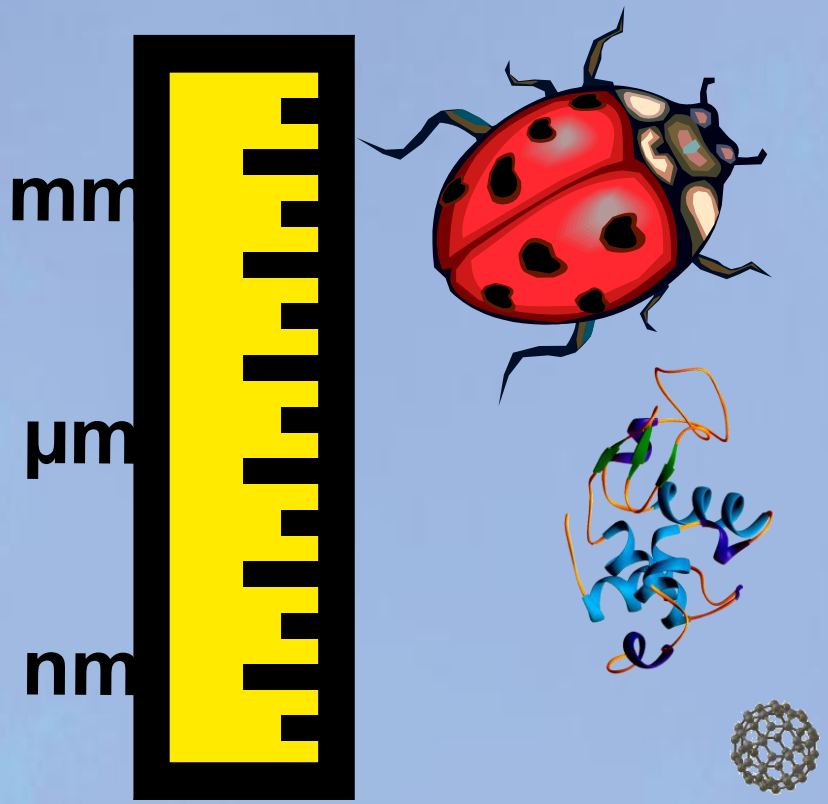
What is Nanotoxicology?

Nanotoxicology is the study of the environmental and human health effects of nanomaterials designed to improve our way of life.



3-step process to characterize nanotoxicity

1. Delivery
2. Chemical/biochemical reaction with target
3. Cellular dysfunction and resultant toxicities

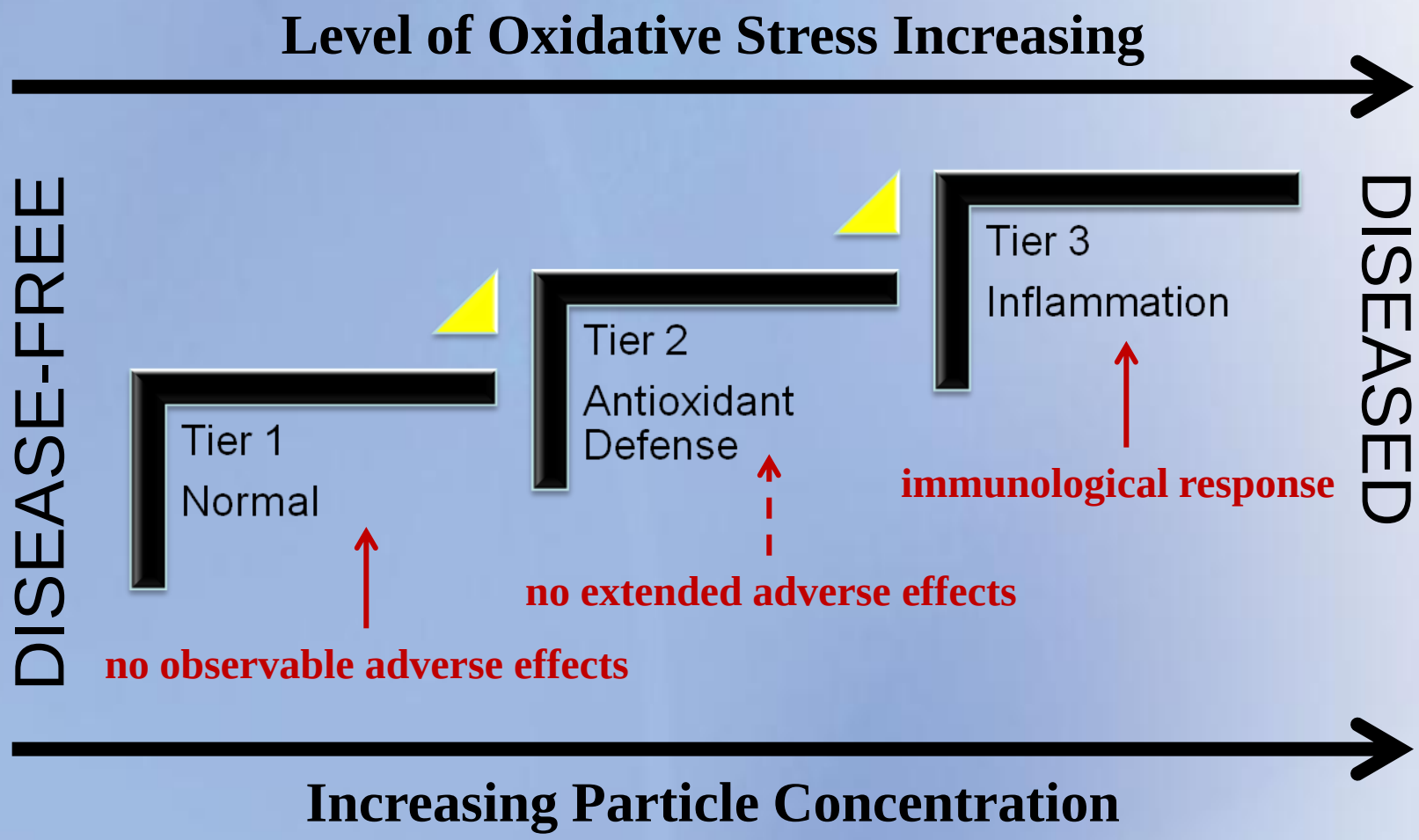
Effects must be related to particle PCC

- Size
- Morphology
- Surface

The most accepted toxicological evaluation is an in vivo study. Because tissues are composed of multiple cell types, in vitro toxicology must use multiple cell types in the study design.

Themes in Nanotoxicology that Influence Other Fields

Approach: The scale of health using the hierarchical oxidative stress model

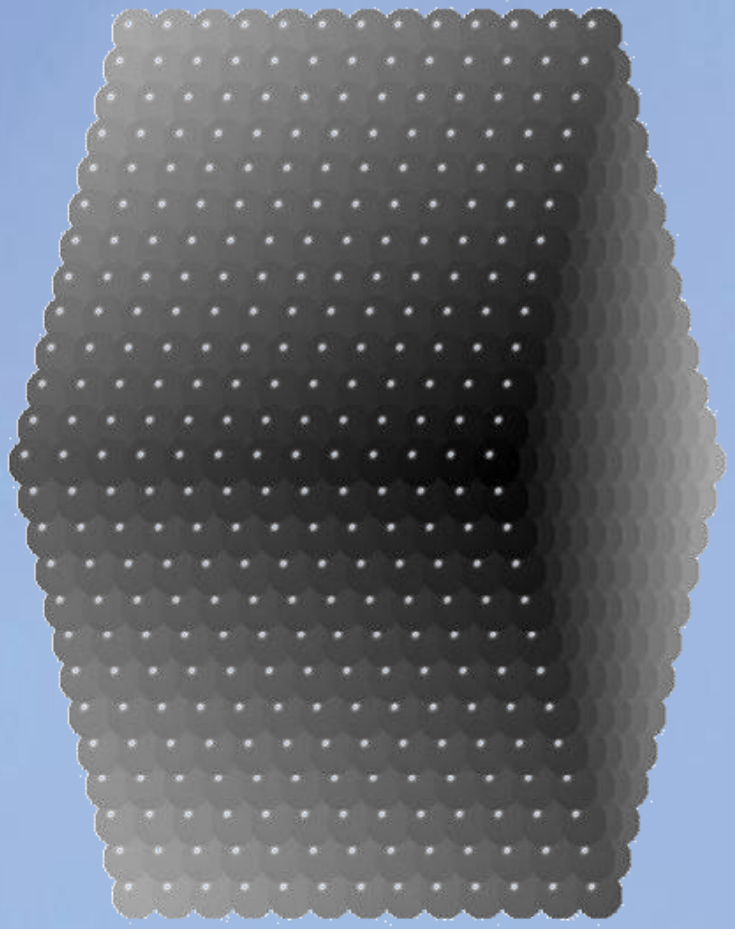


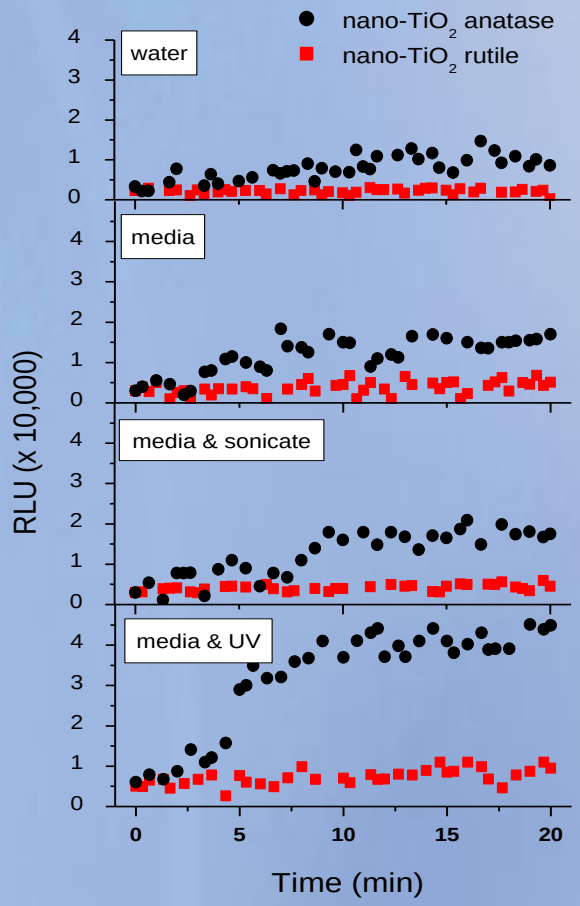
This approach fits well within RTI International

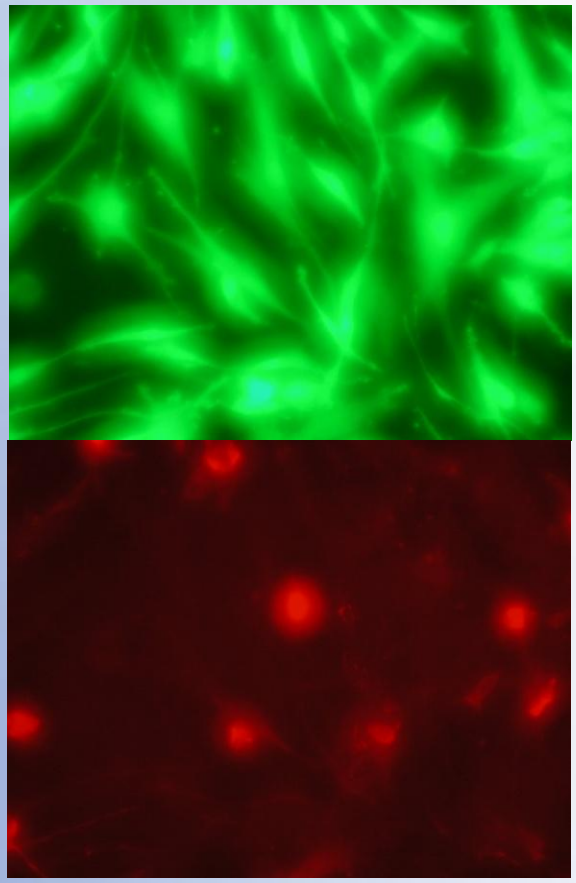
"turning knowledge into practice"

Physical & chemical features of nanomaterials

Structure ➡ Chemistry ➡ Toxicity







Toxicological evaluations require comprehensive material characterization including both physical attributes and chemical surface reactivity.

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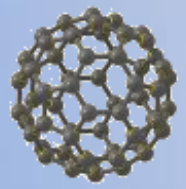
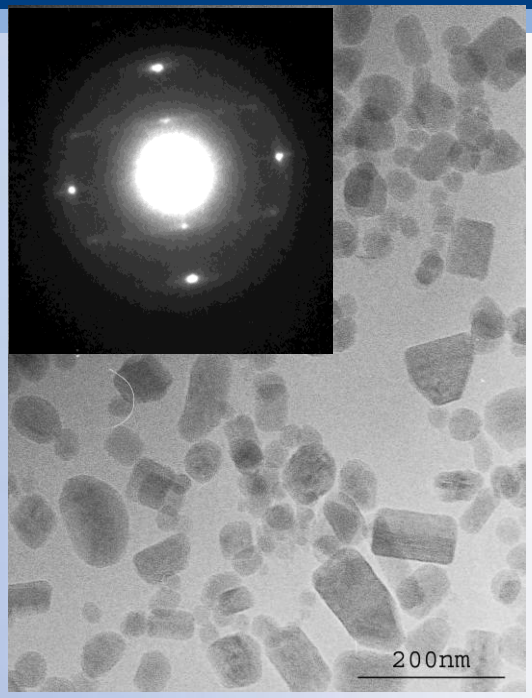
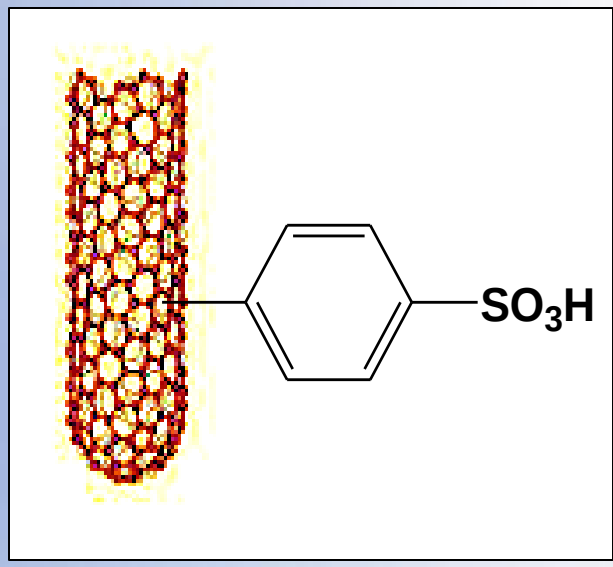
Nanoparticle Properties Relevant To Nanotoxicology





1) Chemical composition
2) Size & size distribution
3) Surface area
4) Surface chemistry, stability, **REDOX**
5) Crystallinity & purity
6) pH & ISP



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A Life-Cycle Approach

Step 1: Material Characterization of Pristine Engineered Nanomaterial

Step 2: Formulate Nanocomposite or Other Nano-Enabled Bulk Material

Step 3: Simulate Wear-and-Tear or Weathering Conditions

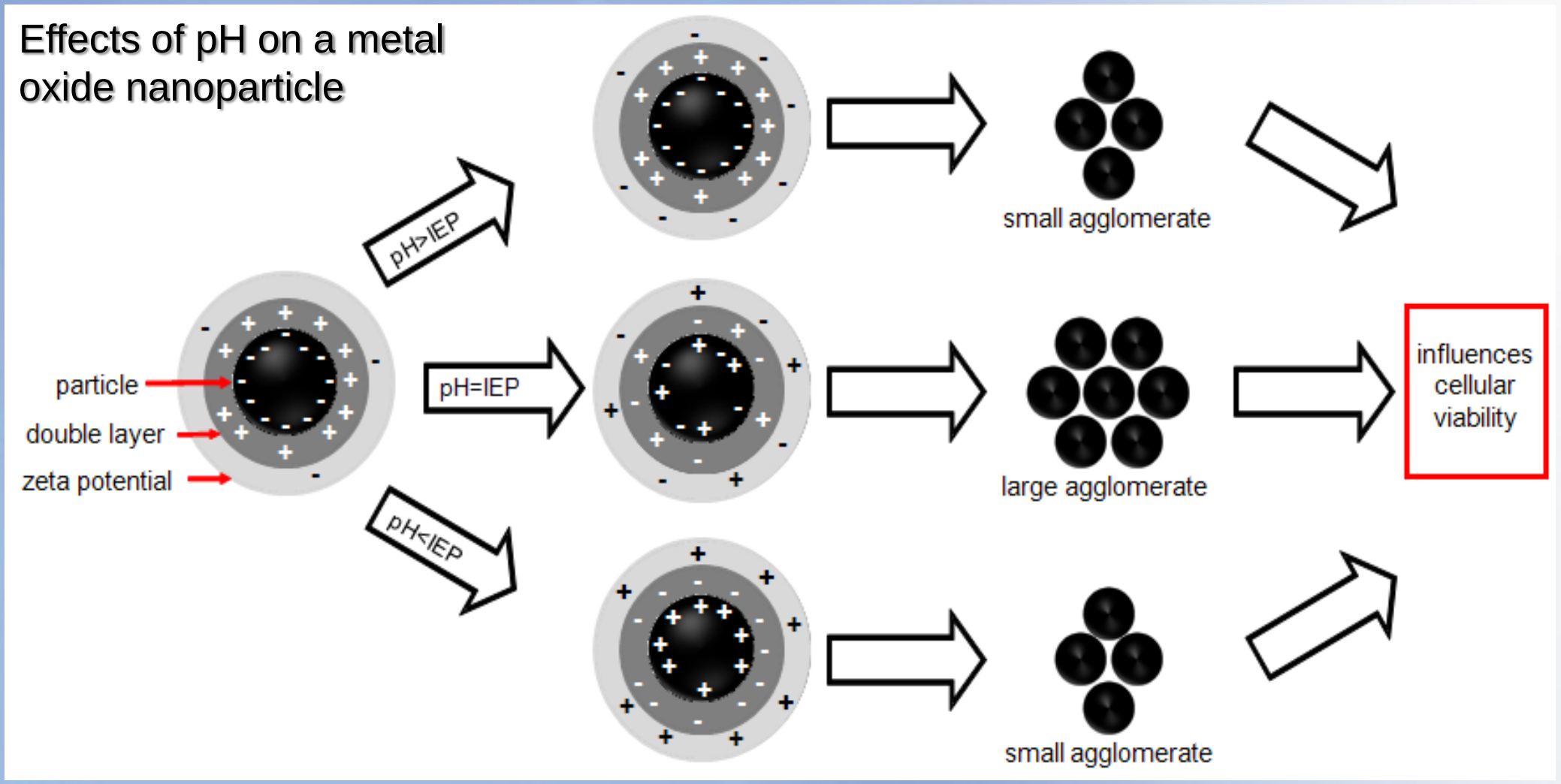
Step 4: Measure Exposures

Step 5: Perform Focused Toxicity Testing

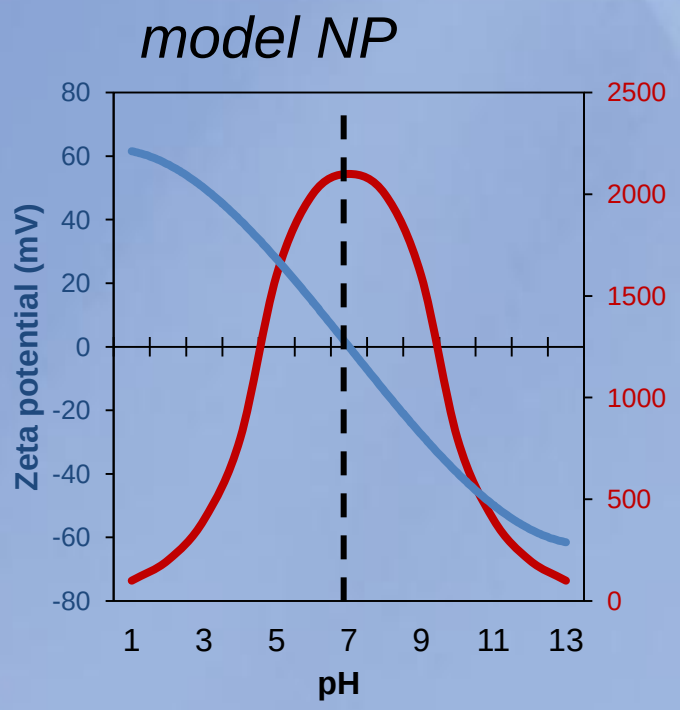
Step 6: Assess and Manage Risks

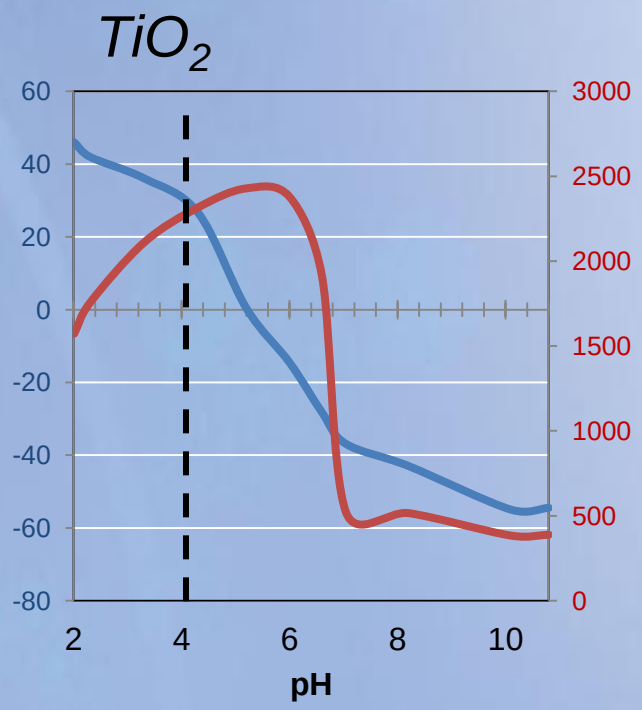
Case Study: Importance of Material Characterization

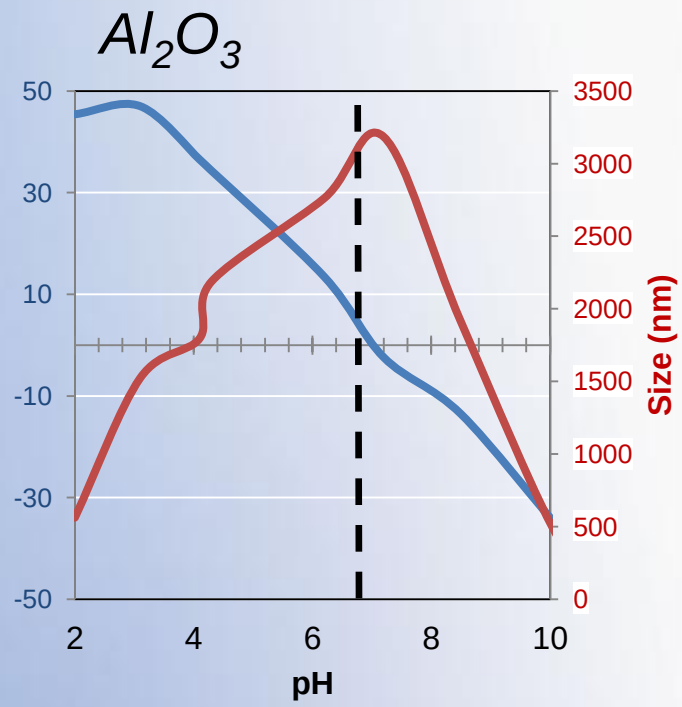
Material Characterization

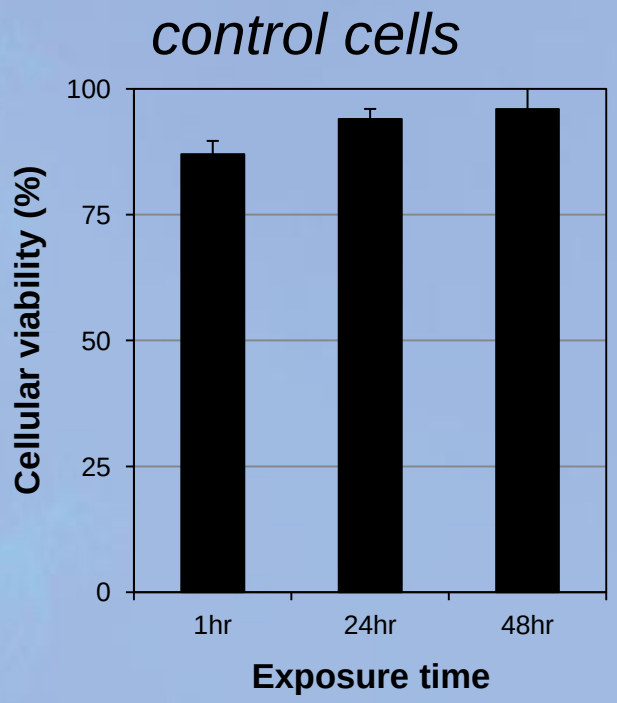


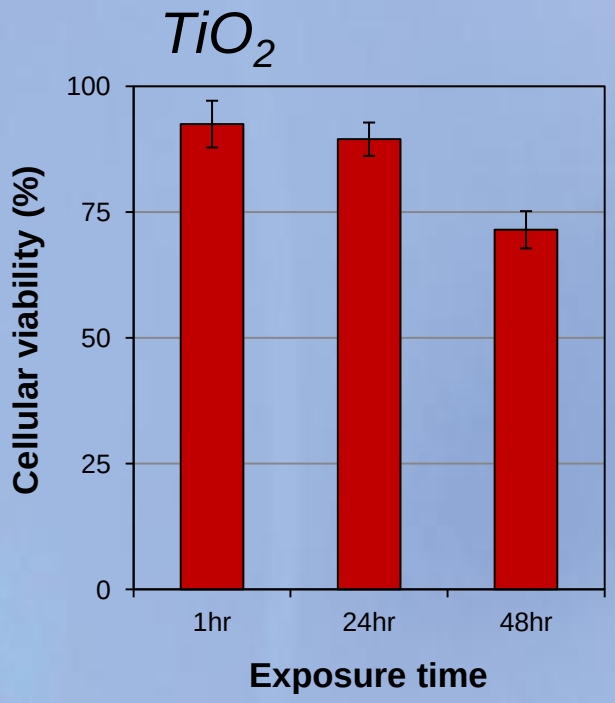
Berg & Sayes, Nanotoxicology, 2009

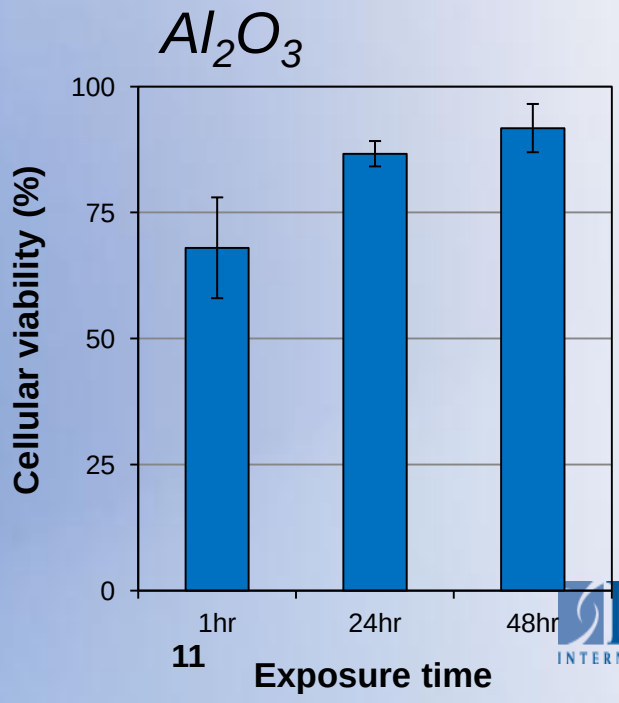












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Material Characterization

Can we predict how nanomaterials would behave in physiological compartments?

	Gastric Acid	Lysosomal Fluid	Intestine & Urine	Blood
pH Level	<2	4.5	5	7.4
Metal oxide nanomaterial	Zeta potential (mV) / Average agglomerate size (nm)			
TiO_2	+46/1573	+22/1860	+7/2390	-37/460
ZnO	+50/360	+44/945	+16/1200	-3/1170
Al_2O_3	+45/561	+38/1750	+27/2400	-4/3050
CeO_2	+32.6/1444	+26/2340	+20/2590	-6/2850
Fe_2O_3	+25.4/1800	-9/1740	-15/1700	-47/830

TiO_2 and Fe_2O_3 nanoparticles demonstrate strongly charged agglomerates at pH=7.4

Case Study: Toxicological Effects (and Mechanistic Analyses) of Silica Nanoparticles

Risk = Hazard × Exposure

Most nanotoxicology studies include hazard identification

only some include exposure assessment

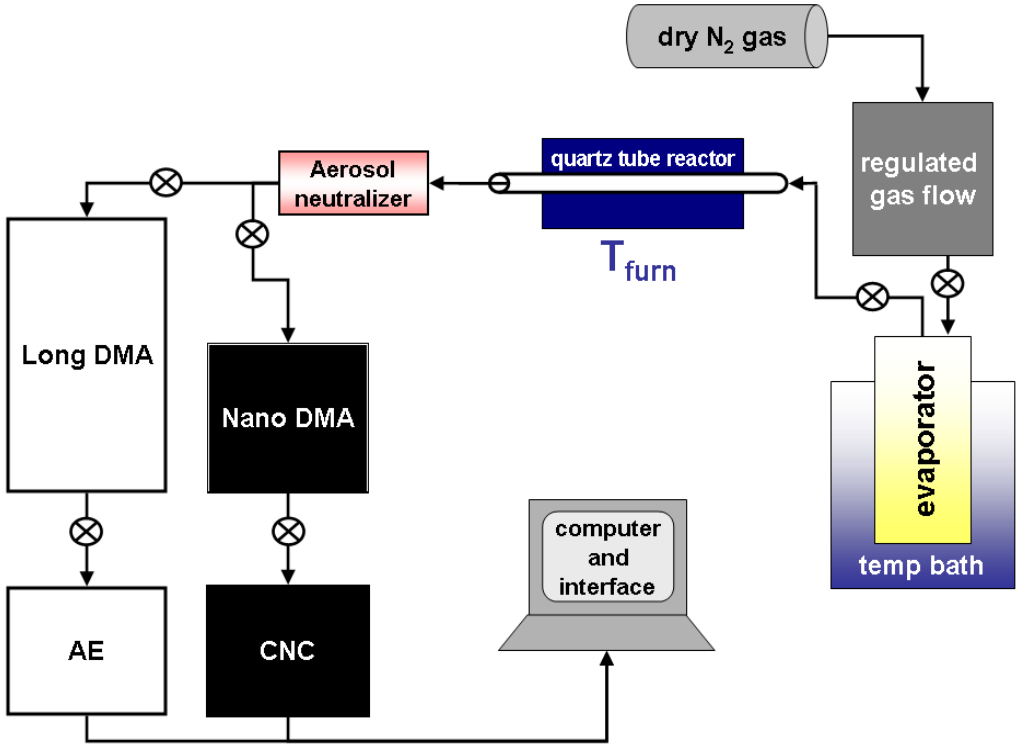
Cannot assume that nanomaterials are the same as their bulk counterpart

but also cannot assume that they are more toxic

Each particle should be tested on a case-by-case basis

In vitro cellular systems will need to be further developed, standardized, and validated (relative to *in vivo* effects) in order to provide useful screening data on the relative toxicity of inhaled particles

Dose Metrics for Inhalation Studies



- Schematic diagram of the aerosol nanoparticle reactor with characterization instrumentation
- TEOS was pyrolyzed to generate SiO_2 nanoparticles that are charged with an aerosol neutralizer
- Characterized with a long or nano DMA, and measured for particle concentration with a condensation nucleus counter or aerosol electrometer

Experimental Design

Exposure Groups

- Group 1 (3 day exposure)
  - Sham (5 rats/group)
  - Particle-exposed (5 rats/group)
    - *Targeted particle sizes = 35 nm and 80 nm*
- Group 2 (1 day exposure)
  - Sham (5 rats/group)
  - Particle-exposed (5 rats/group)
    - *Targeted particle sizes = 35 nm and 80 nm*

Inhalation

Post-exposure evaluation via lung fluid and lung tissue

24 hr ***1 wk*** ***1 mo*** ***2 mo***

← (lung fluid) → (tissue)

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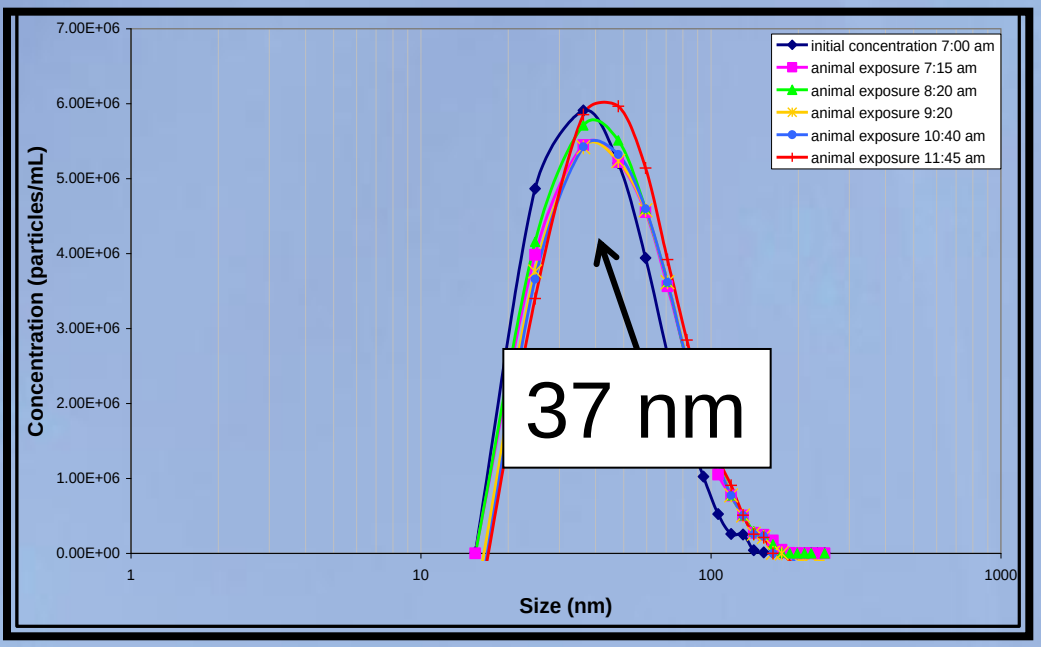
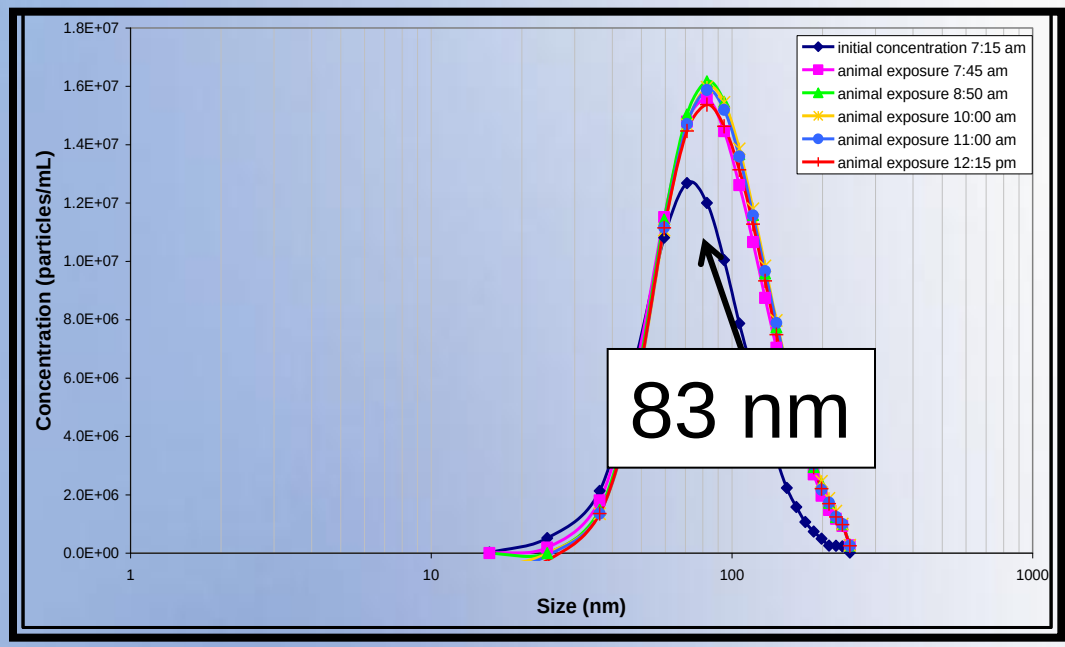
Particle Physicochemical Characterization

Aerosol nanoparticle size distributions for SiO_2 exposure in the inhalation chamber as a function of exposure time demonstrating aerosol stability





Typical aerosol exposure run on day 1 for the d_{50} = 37 nm particle generation experiment

Typical aerosol exposure run on day 3 for the d_{50} = 83 nm particle exposure experiment

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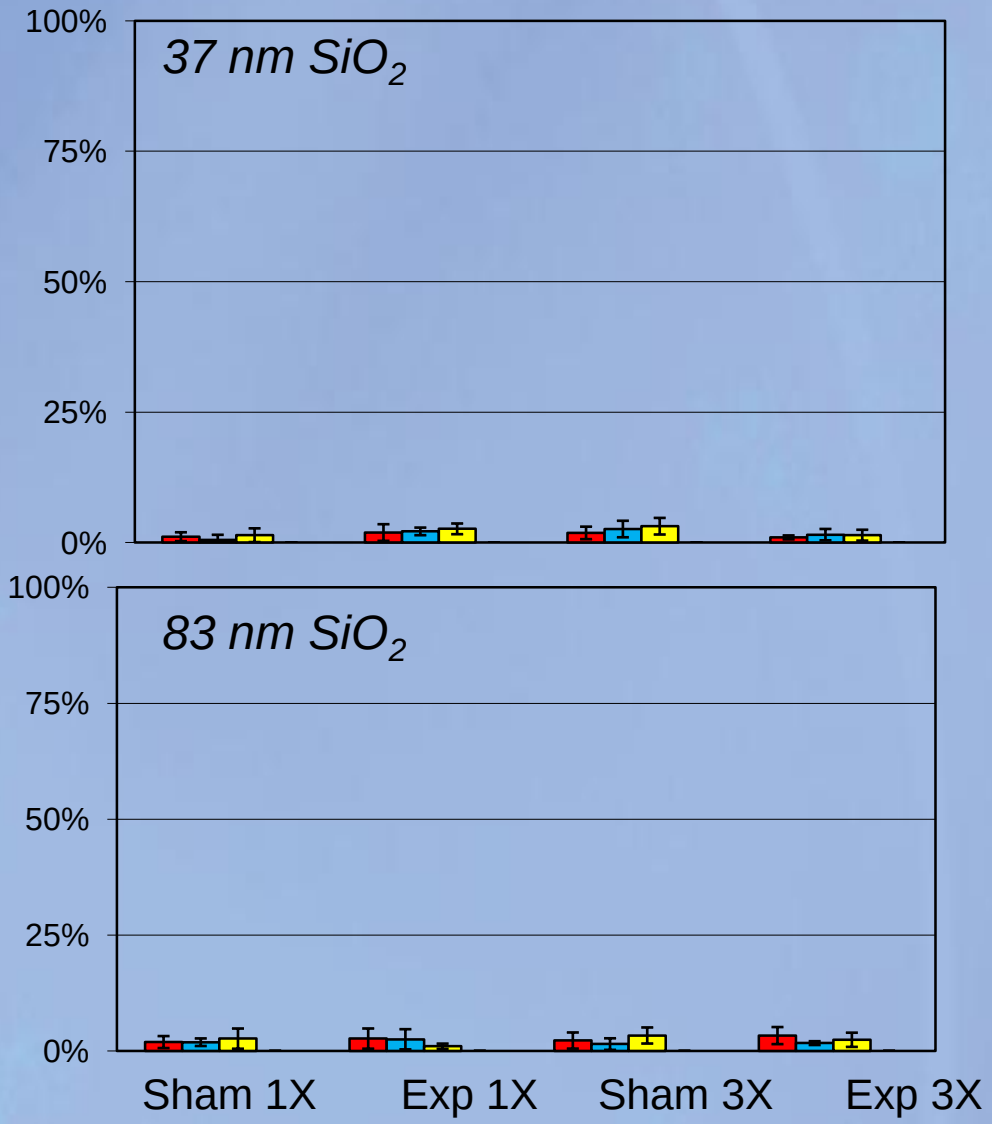
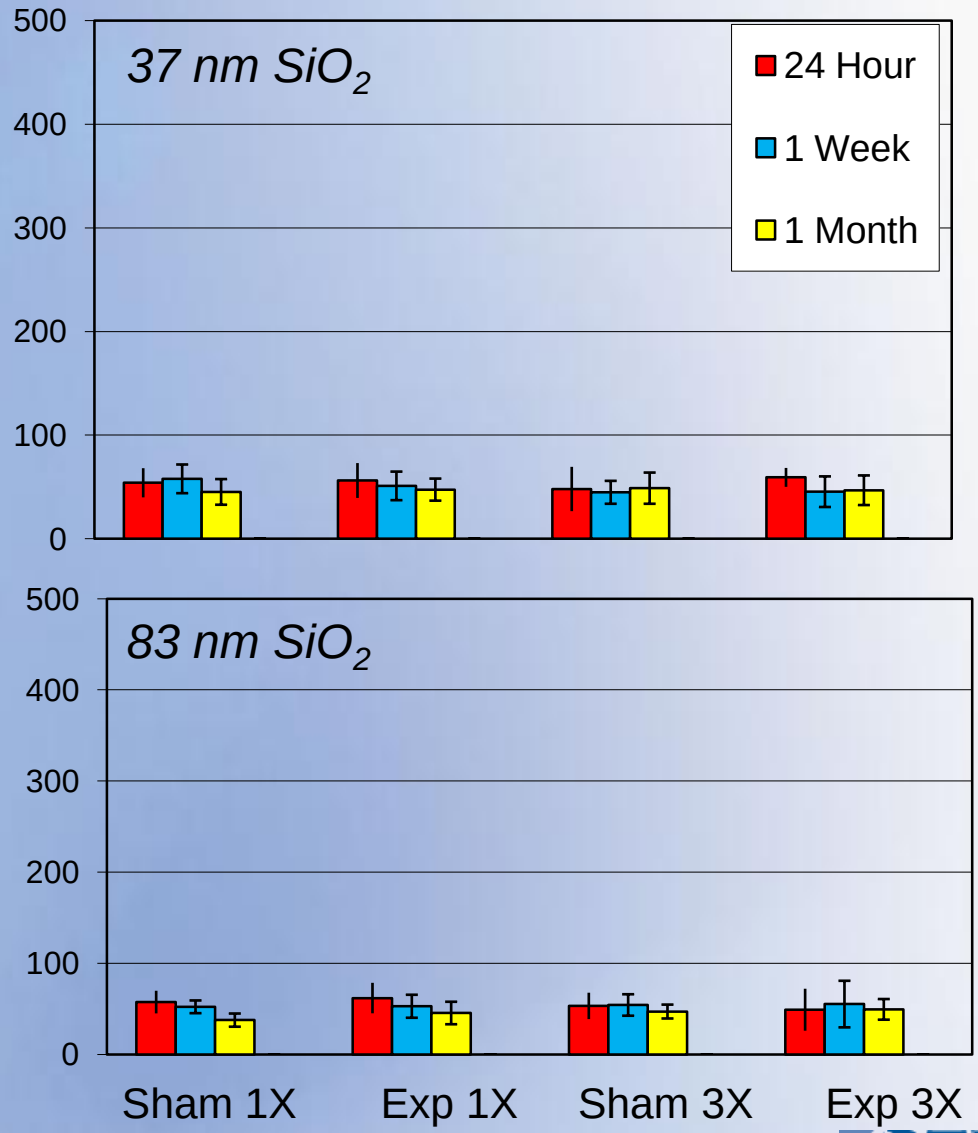
Pulmonary Effects

Inflammatory Response



Lung lavage fluid percent polymorphed neutrophil (%PMN) values

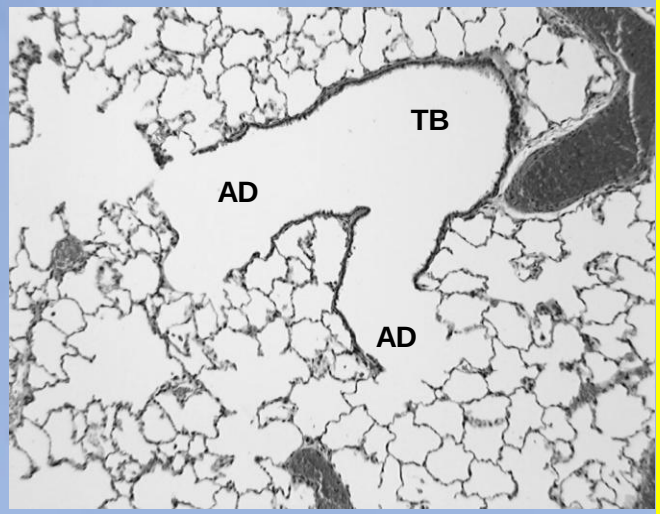
Dead Cells



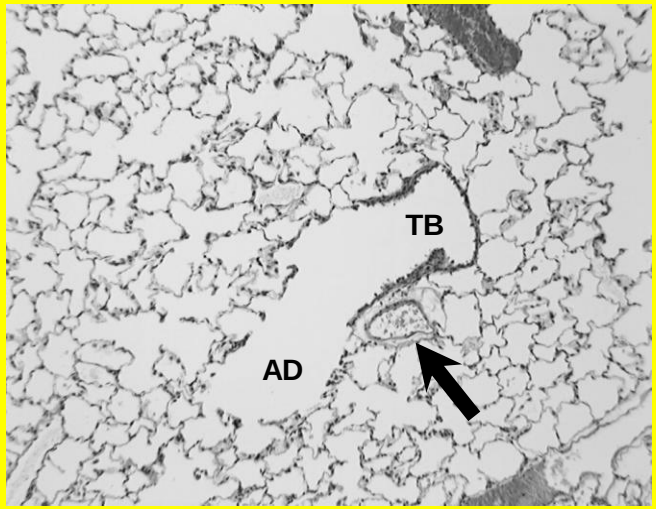
Lung lavage fluid lactate dehydrogenase (LDH) values

Pulmonary Effects

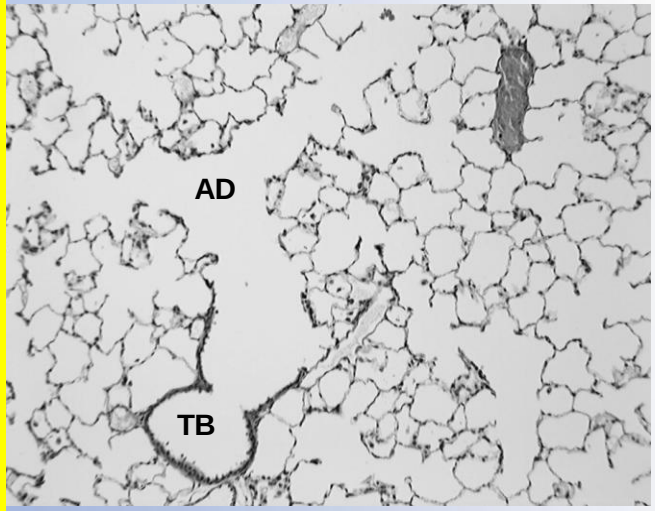
Lung pathology after 2 month exposure



sham (unexposed) animals



animals exposed to
37 nm aerosolized silica
nanoparticles



animals exposed to
83 nm aerosolized silica
nanoparticles

	Exposed	Sham
Catalase Activity ($mU \cdot mL^{-1} \cdot mg\ protein^{-1}$)	34.35 ± 0.2	31.63 ± 0.01
Total Glutathione (μM/10k cells)	2.34 ± 0.19	1.69 ± 0.14

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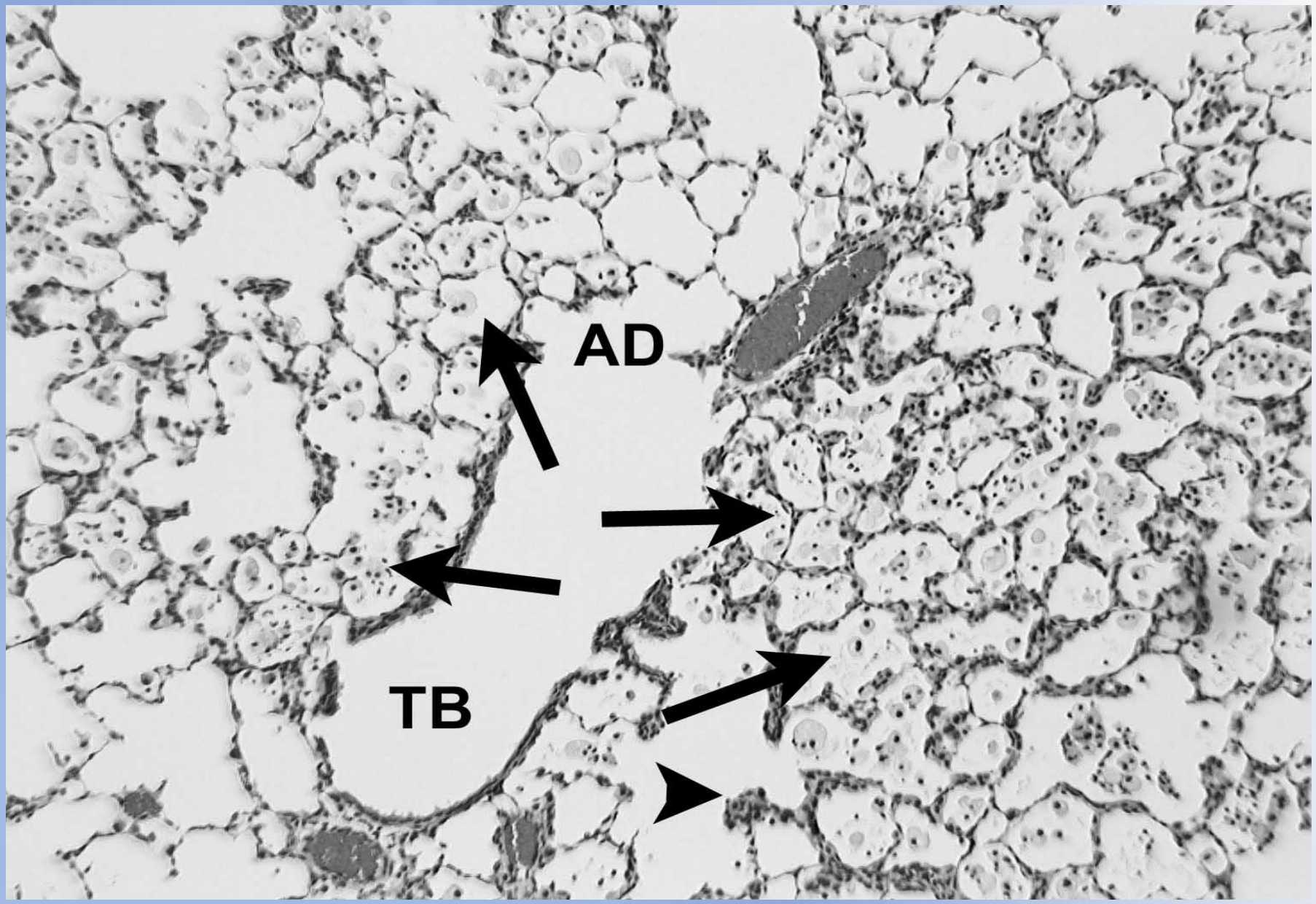
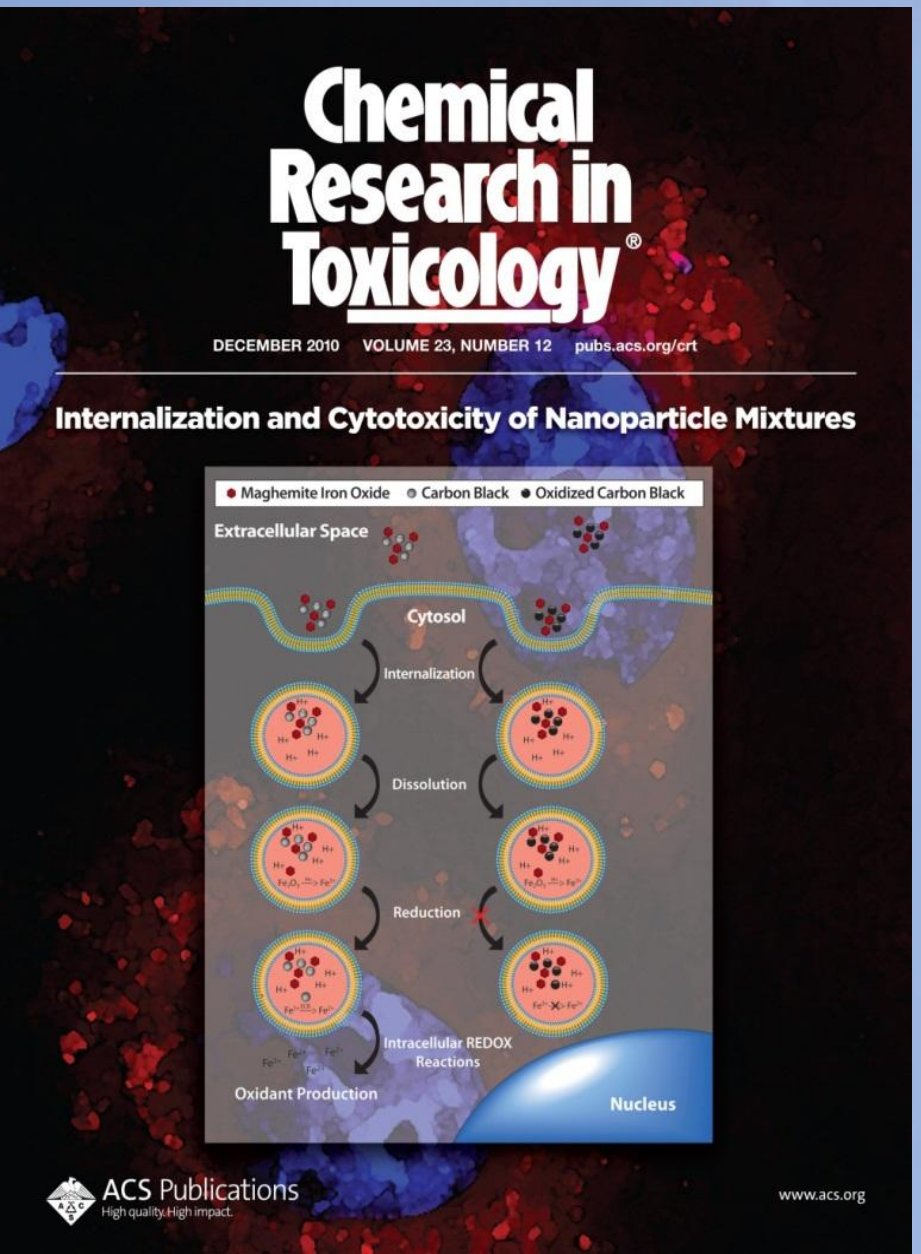
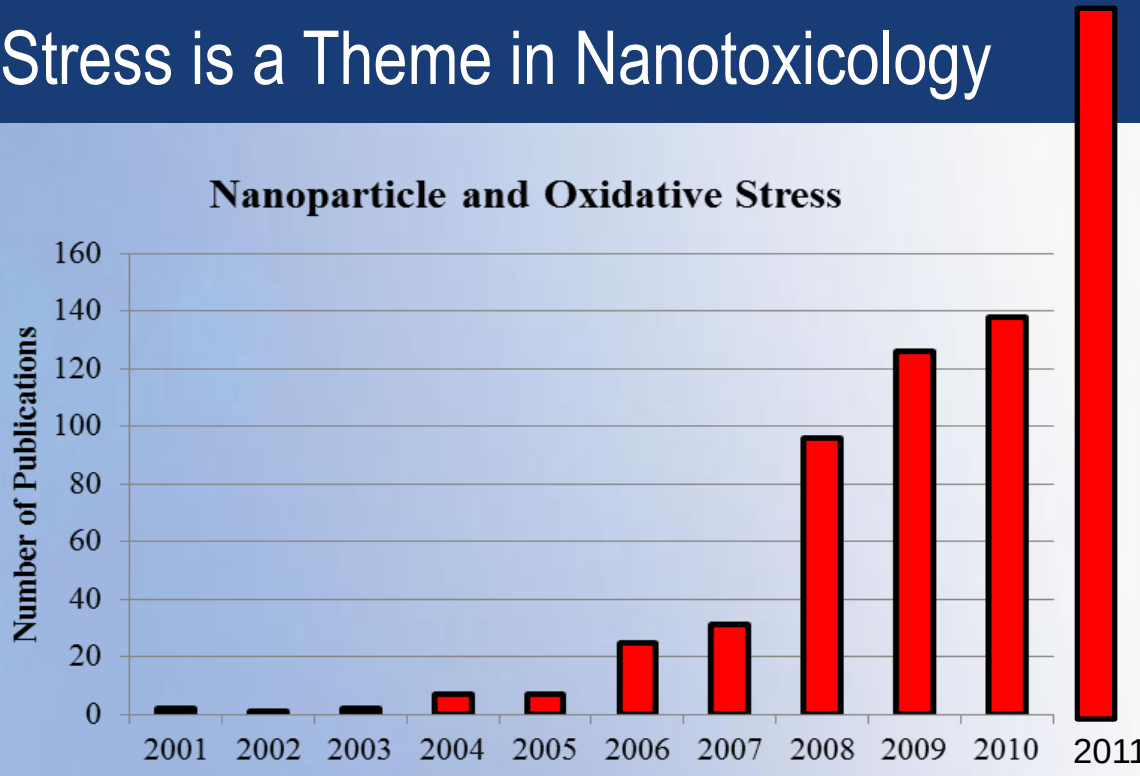
Lung Tissue of Rat Exposed to Positive Control Particle after 1 week

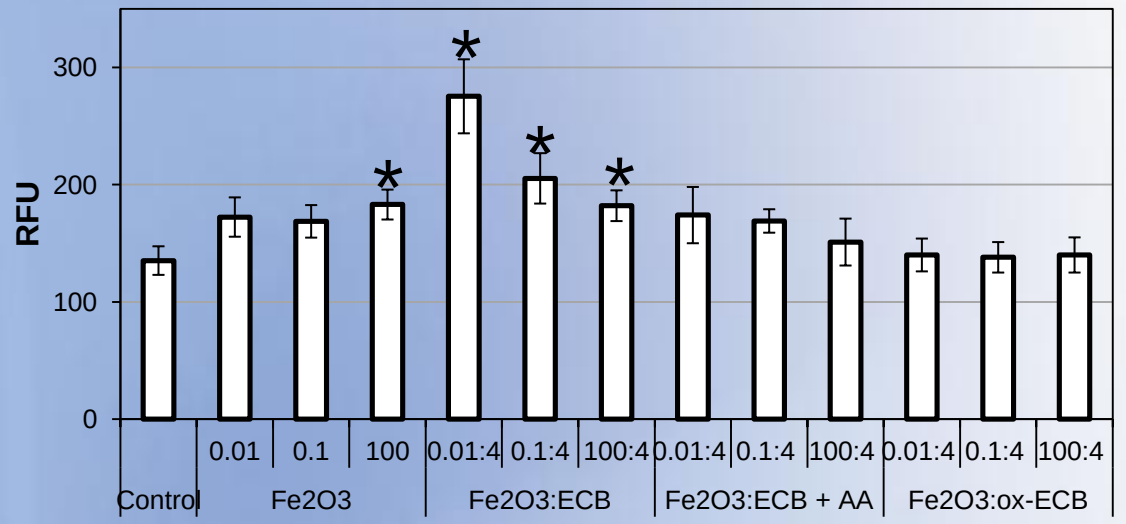


Oxidative Stress is a Theme in Nanotoxicology

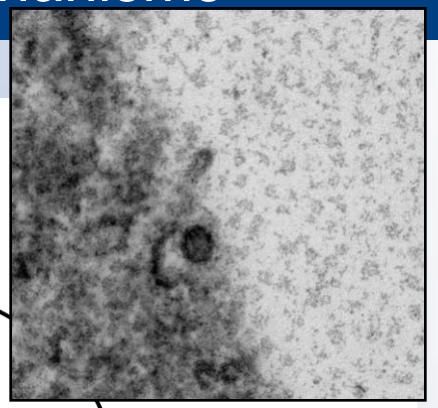
Berg and Sayes, CRT (2010)

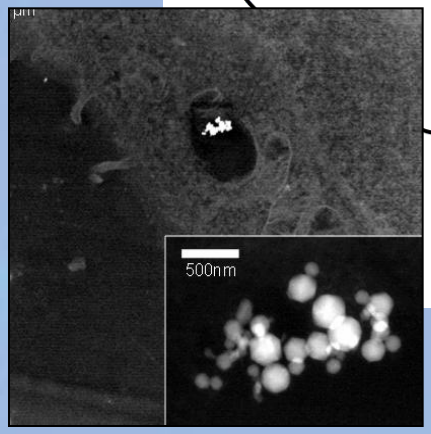






Differential Cellular Uptake Mechanisms

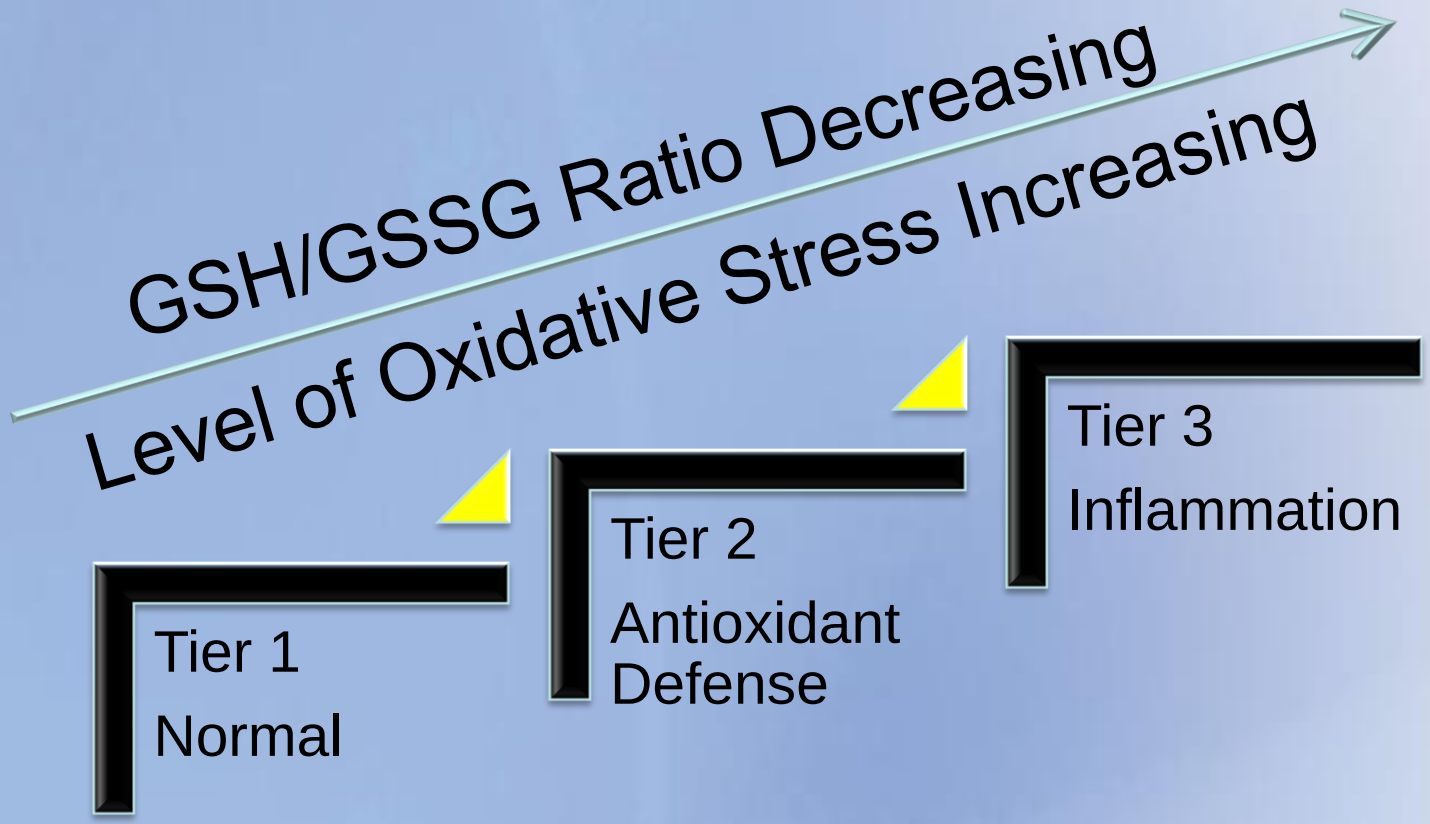




The Role of *In Vitro* Toxicology in Nanotoxicology

Due to the enormous range of nanomaterial-types, coupled with the infinite variety of surface coatings, *in vitro* toxicology will play a major role in hazard identification

Hierarchical Oxidative Stress Model



But, are we considering if the effects are reversible?

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Characteristics of A549 and MeT-5A Cells

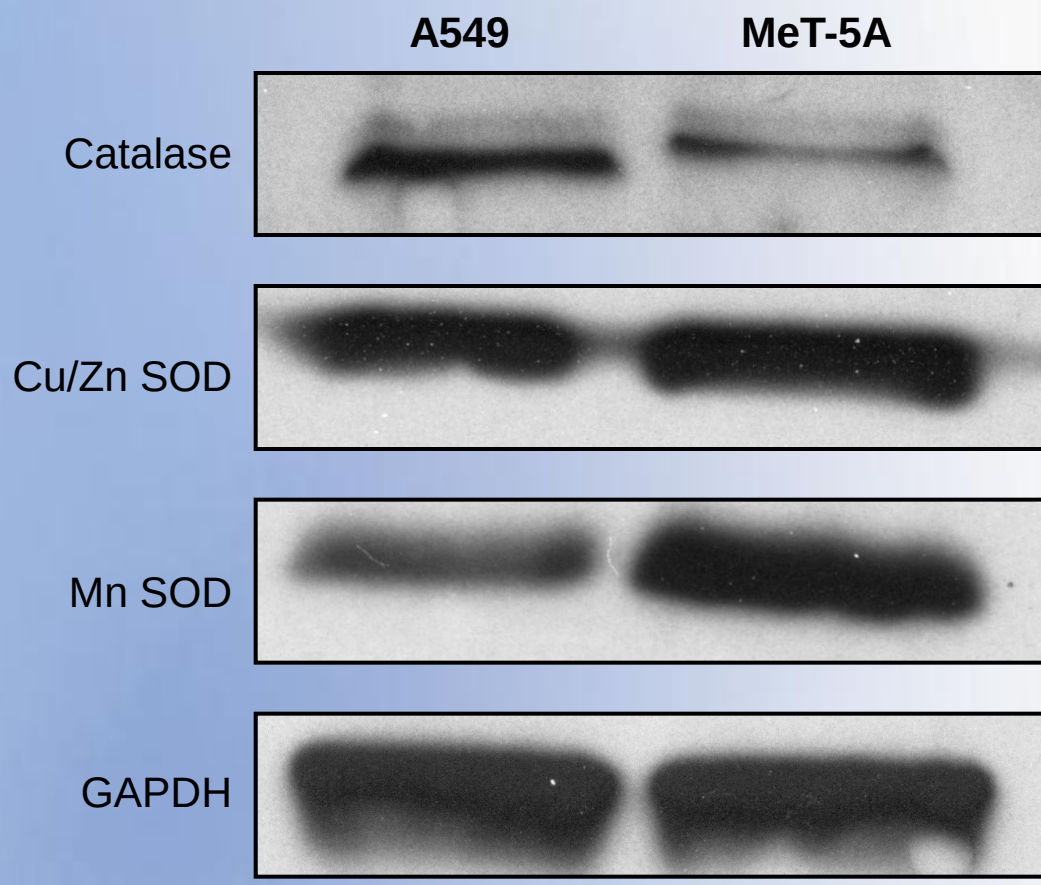
A549 Cell Line

- Isolated through explant culture of lung carcinomatous tissue
- Considered a type II lung epithelial cell epithelial (surfactant producing)
- Reported to have high levels of antioxidant enzymes

MeT-5A Cell Line

- Isolated from pleural fluids obtained from non-cancerous individuals
- Transfected with a plasmid containing SV40
- Normal cell precursor to mesothelioma

Berg JM, Figueroa DE, Romoser AA, Sayes CM. (2012) *Toxicology In Vitro*, submitted.



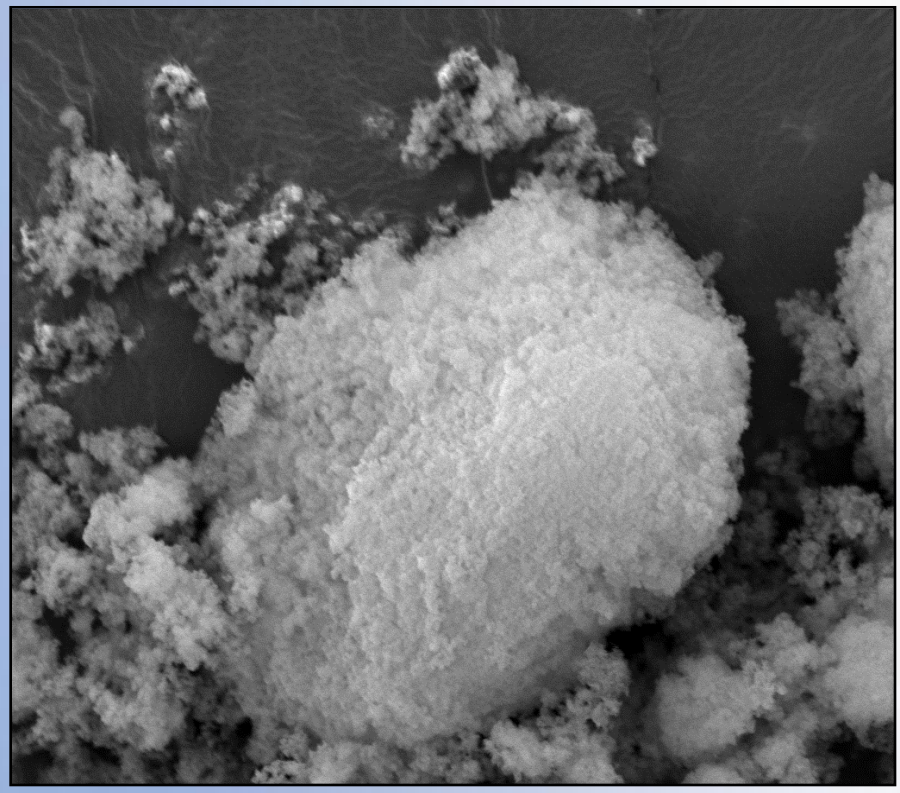
Silica at the Nanoscale: How safe is it?

Micrometer amorphous SiO_2 is under GRAS classification and often used as a negative control in toxicological studies

Micrometer crystalline SiO_2 is a Class II IARC probable carcinogen often associated with respiratory diseases such as:

- Silicosis
- Fibrosis

Endpoints in toxicological studies involving SiO_2 are often dependent upon particle surface characteristics

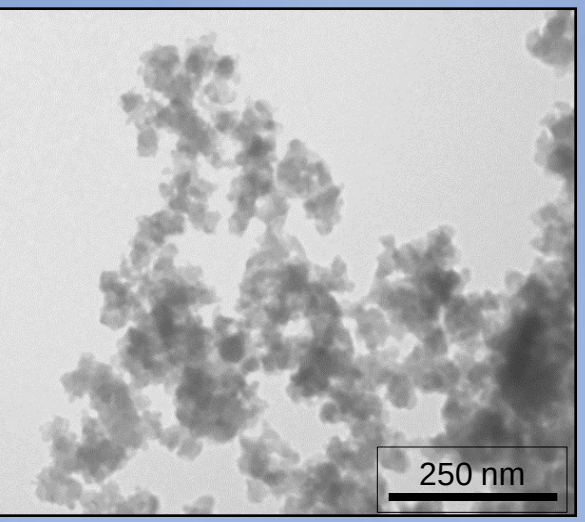


SEM: Nanoscale Amorphous SiO_2

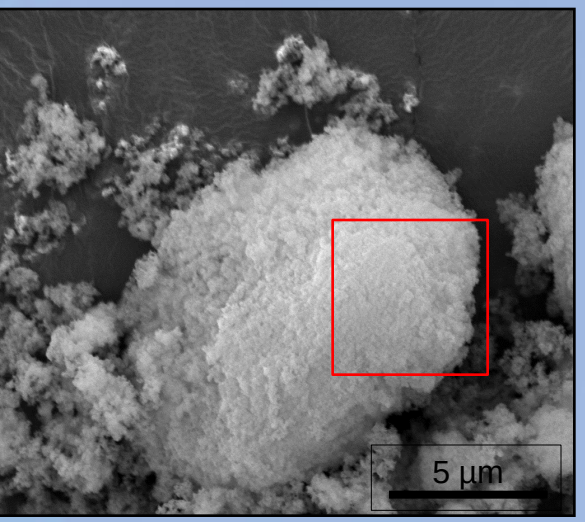
Since SiO_2 particle toxicity has often been reported as a function of surface parameters, and since nanoparticles exhibit high surface area to mass ratios, it is necessary to reevaluate the safety of SiO_2 particles at the nanoscale

Silica Nanoparticle Characterization

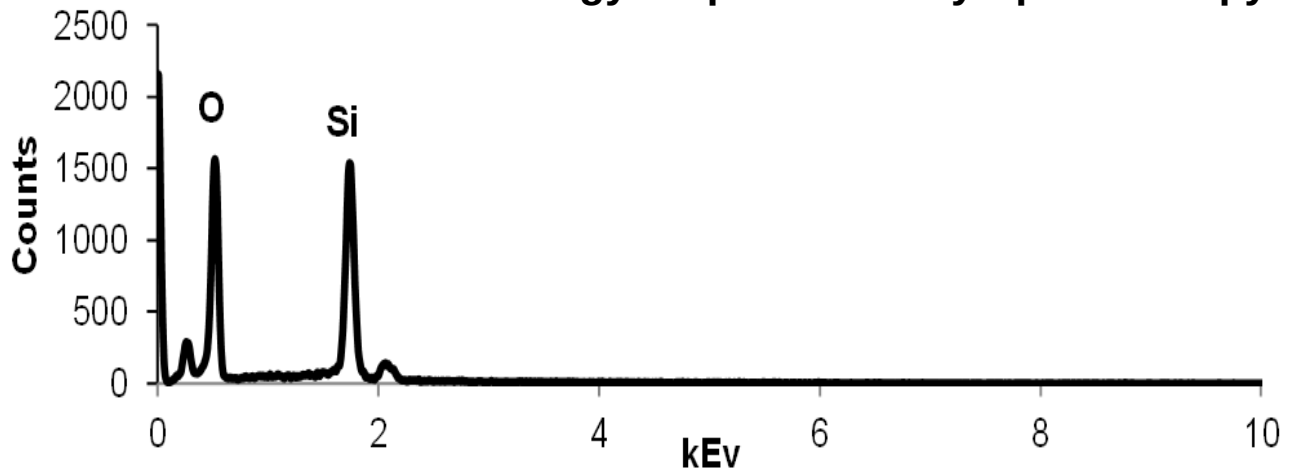
Transmission Electron Micrograph



Scanning Electron Micrograph



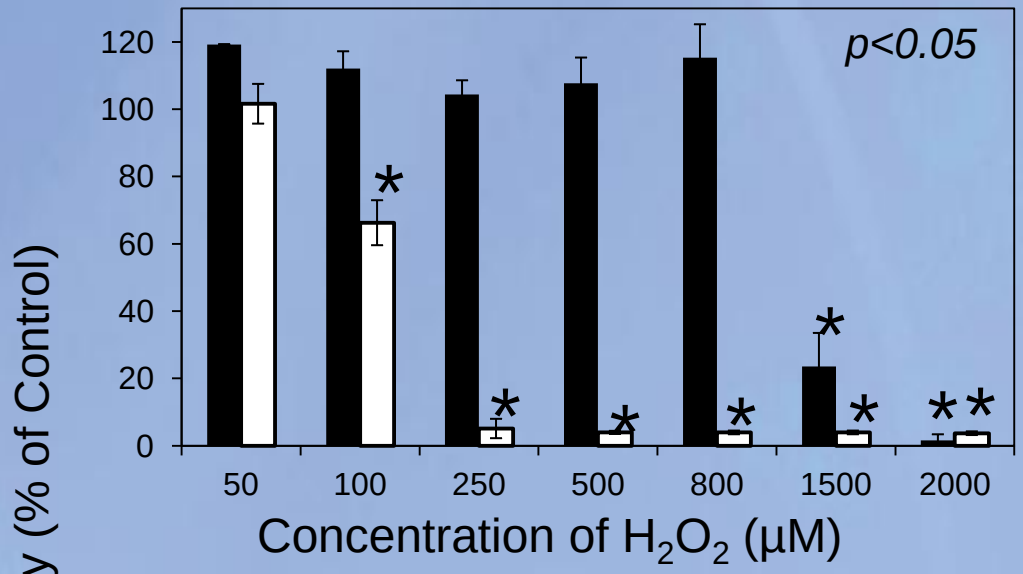
Energy Dispersive X-ray Spectroscopy



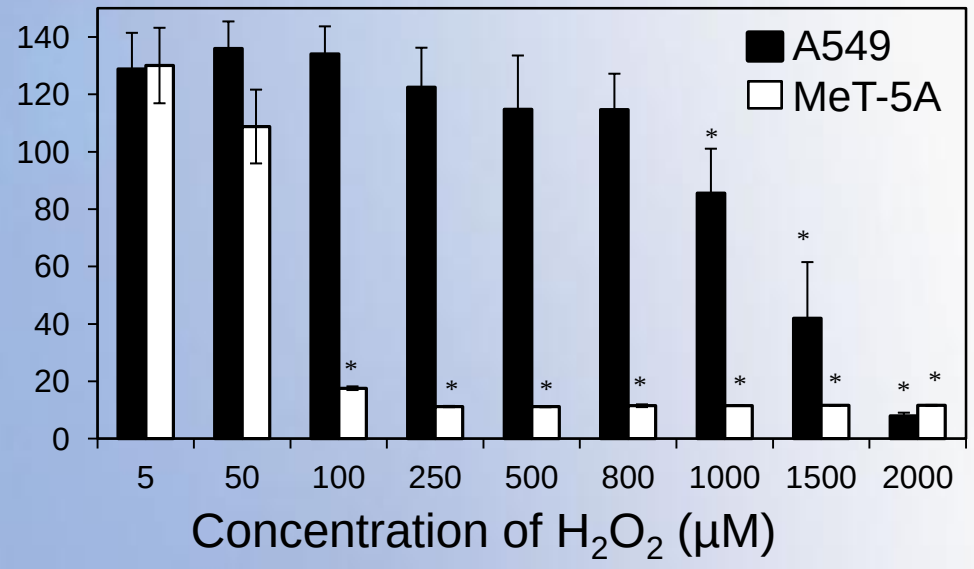
Physicochemical Property	SiO_2
Manufacture's Reported Size (nm)	15
Actual Primary Particle Size (nm)*	33.5 ± 7.73
Zeta Potential (mV)	-47.6 ± 5.39
Crystalline State	Amorphous
Density (g/cm^3)	1.948 ± 0.0065
Specific Surface Area (m^2/g)	576.23

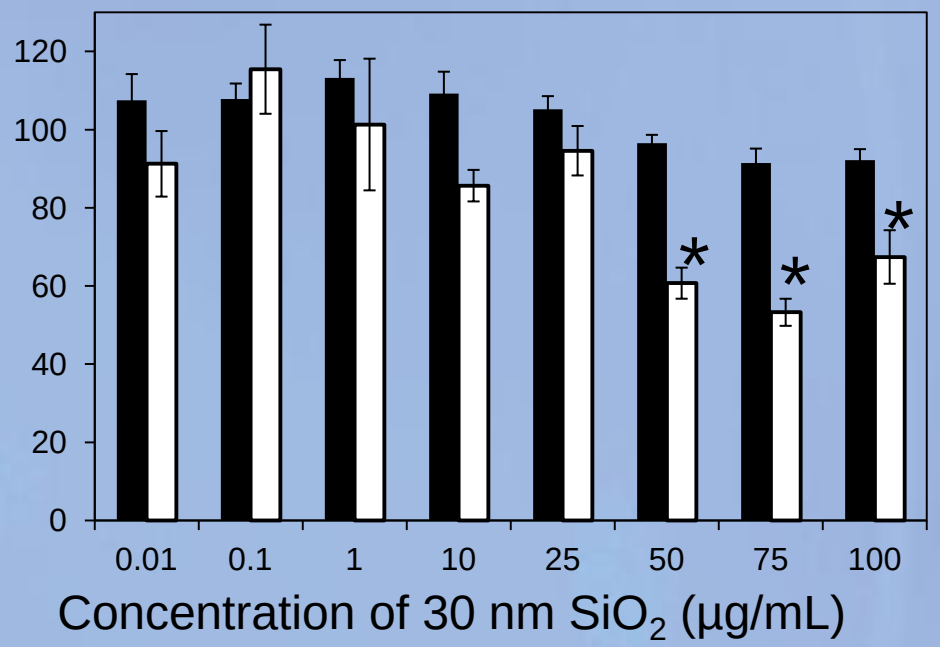
Differential Response to Oxidative Stress

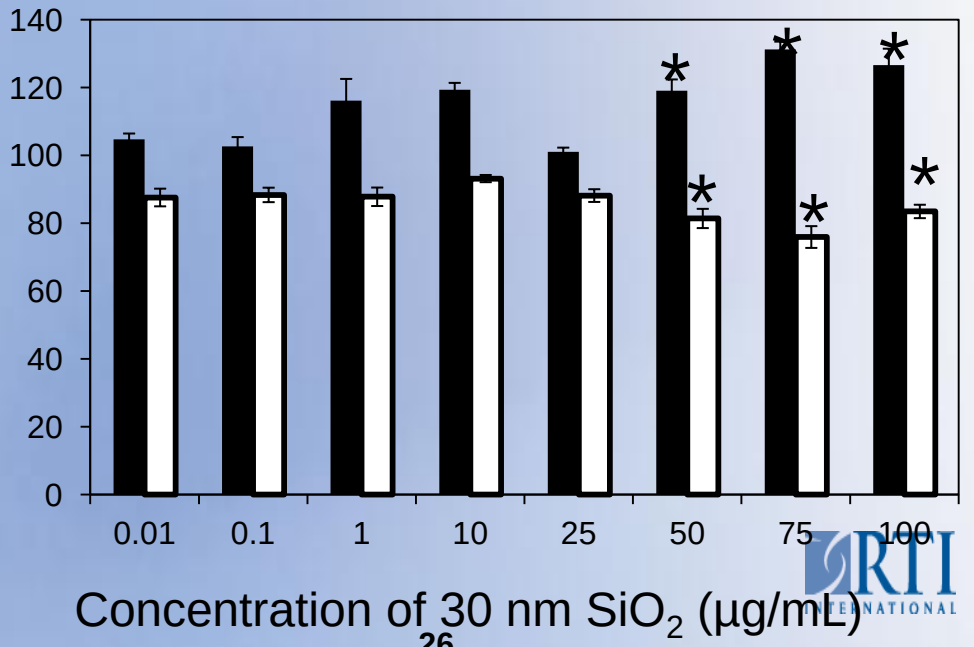
MTT Assay



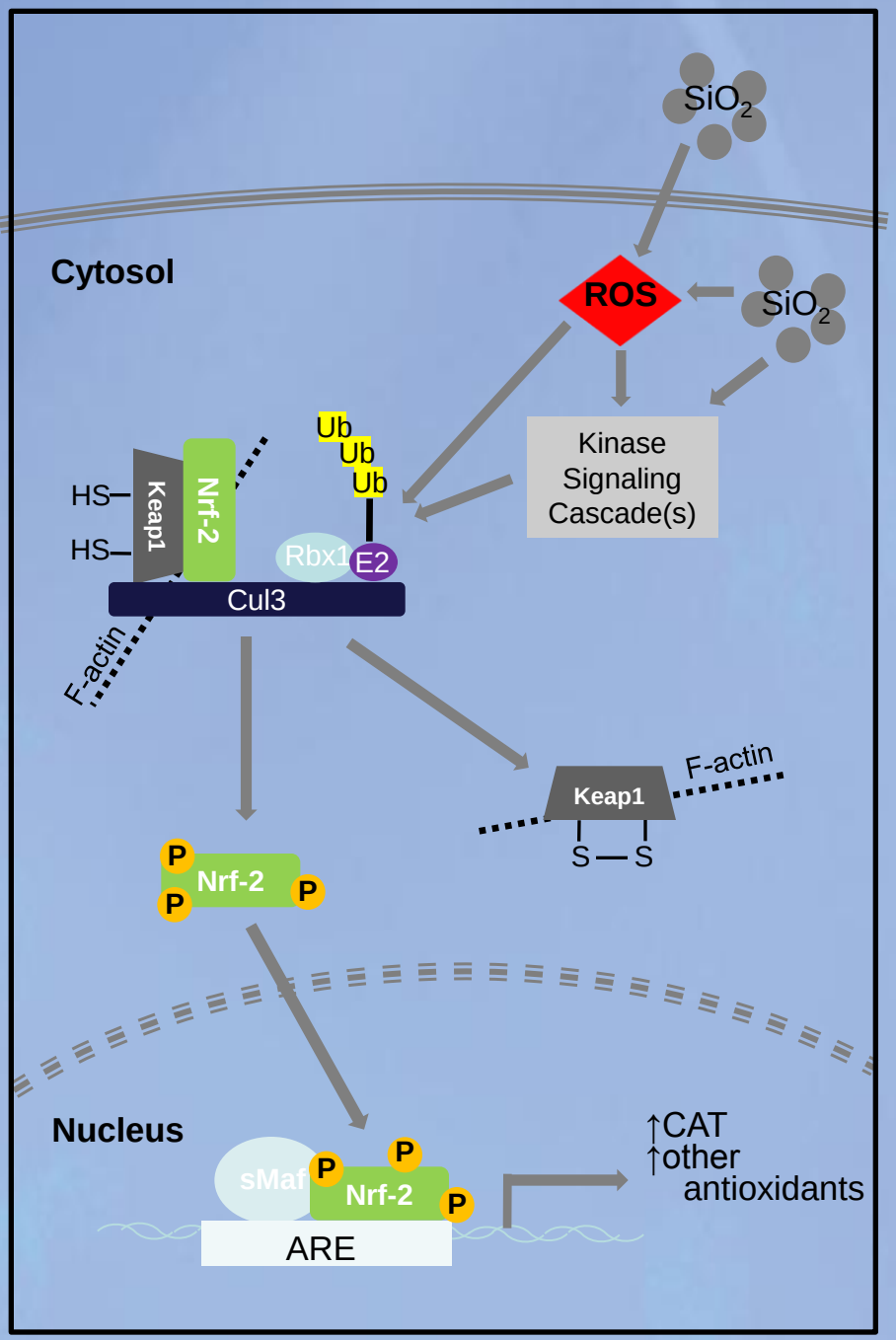
Live:Dead Cell Counts







NF-E2 Related Factor (Nrf2)



The Good

- Critical regulator of intracellular antioxidants and phase II detoxification enzymes.
- May be induced by:
  - Electophilic Stress
  - Kinase Signaling Pathways
- Activation of Nrf2 can be cyto-protective at low levels of ROS

The Bad

- Elevated Nrf2 is a major obstacle to the successful treatment of many cancers

The Ugly

We hypothesize that SiO_2 nanomaterials, which has been shown to produce ROS, may activate the Nrf2 signaling pathway. This induction might lead to the induction of many phase II genes which can have implications in both resistance against cell death and carcinogenesis

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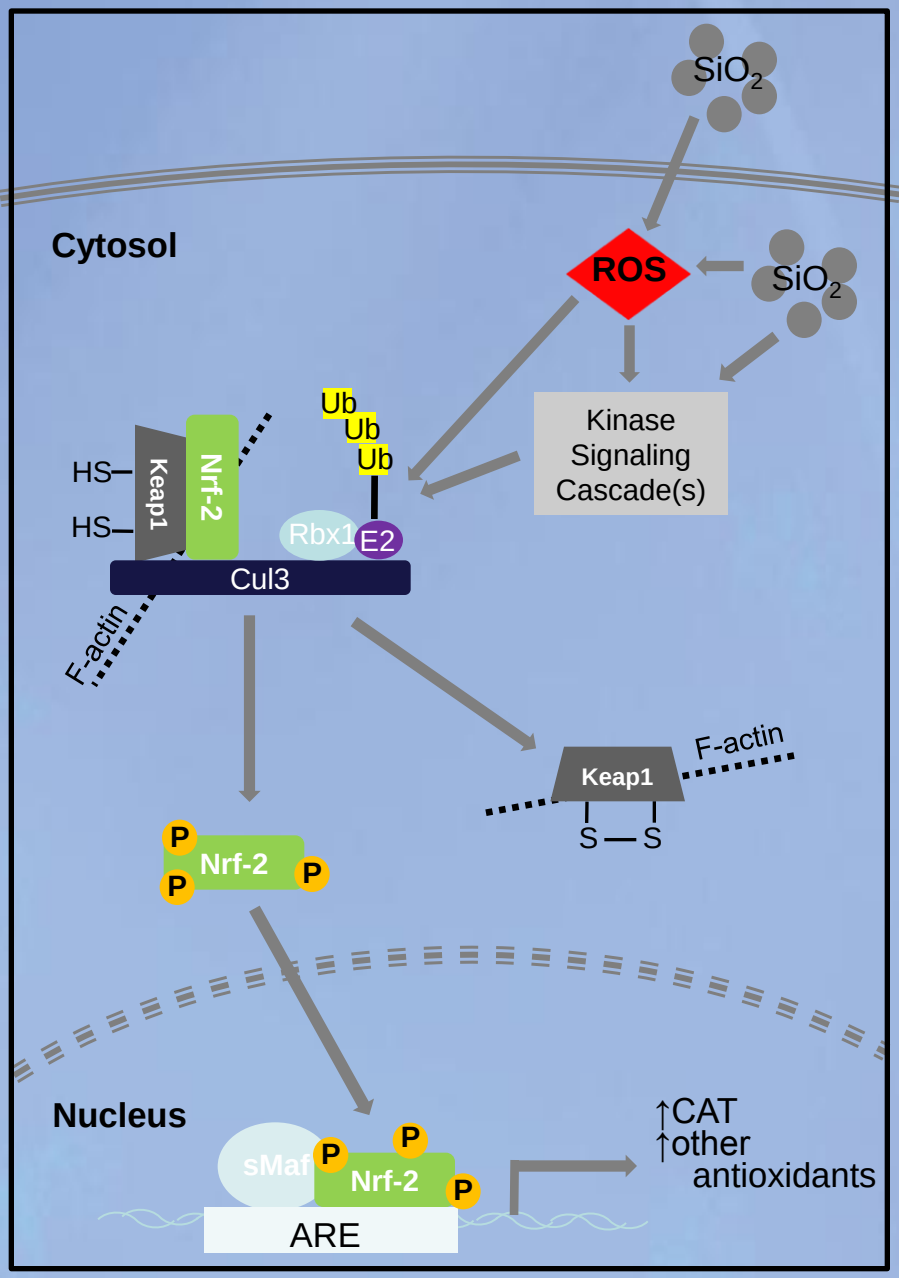
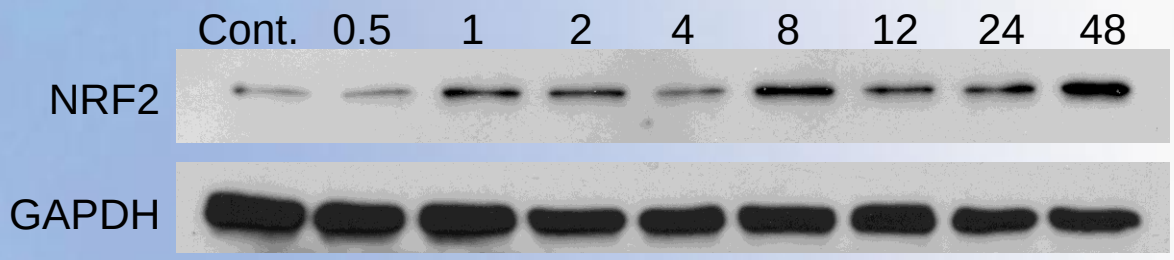
Nrf2 Stabilization

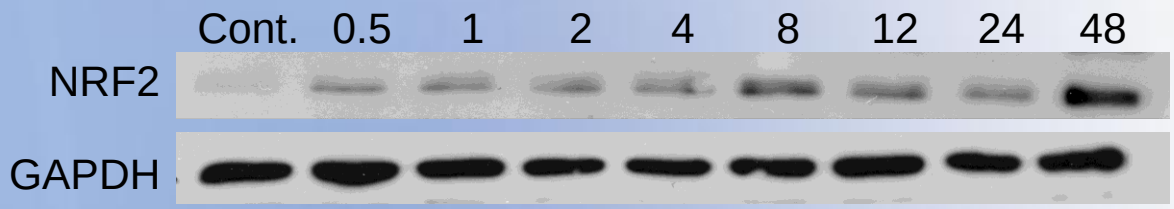


SiO_2 exposed epithelial cells

values given in hours



SiO_2 exposed mesothelial cells



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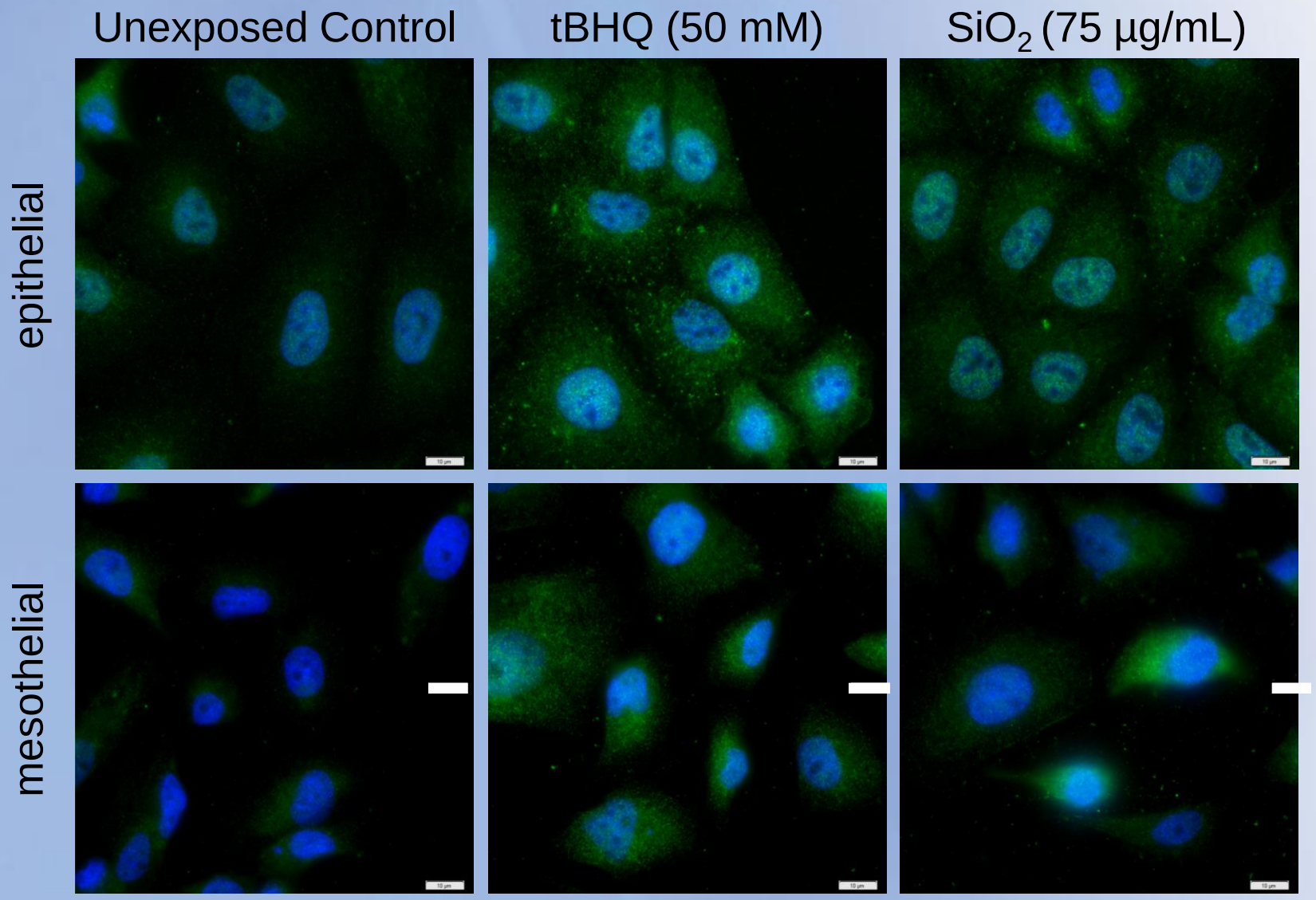
Nrf2 Cytoplasmic Stabilization & Nuclear Translocation

Cytoplasmic stabilization & nuclear translocation of NRF-2 (GREEN) was evident following treatment with tBHQ or SiO_2 nanoparticles for 24 hrs in both cell lines



Scale bar = 10 mm

Case Study: Mixtures Nanotoxicology – its hardly ever just one

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Exposure to Nanoparticle Mixtures

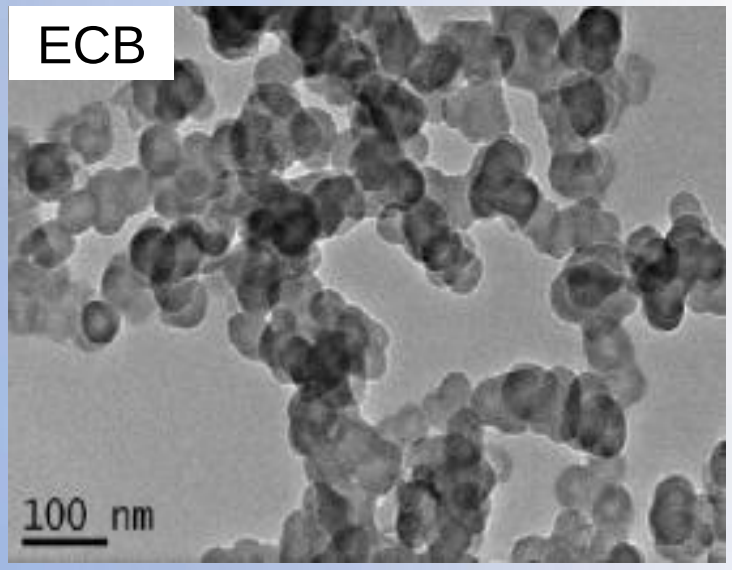
Reported Exposure to Nanoparticle Mixtures	Reference
Manganese from nearby welding during $Li_4Ti_5O_{12}$ handling	Peters, 2009
Iron and nickel as catalysts in carbon nanotube synthesis	Maynard, 2004
Silicon & asbestos from insulation during CNTsynthesis	Han, 2008
Combustion-derived particles from forklifts, heaters, & traffic	Kuhlbusch, 2006 & 2010

Therefore, nanoparticle exposures are often mixtures of combustion derived
carbonaceous particles and **transition metals**

Nanoparticles Representative of Mixtures

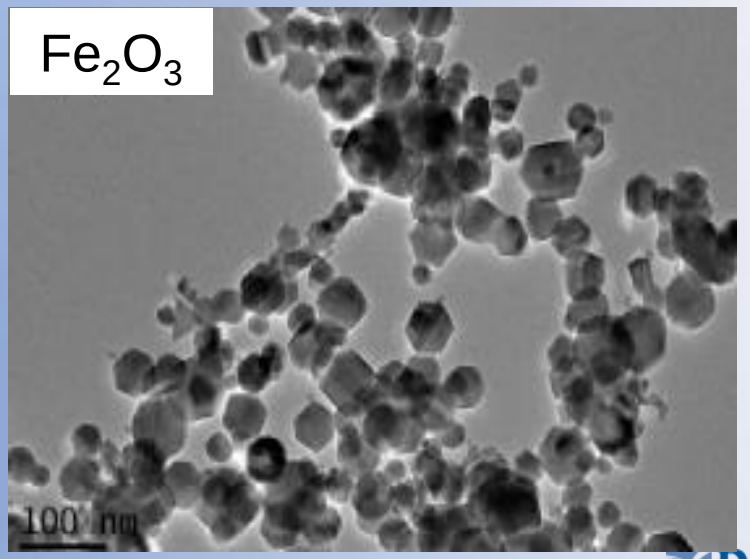
Carbonaceous Particle

- Engineered Carbon Black
- Used as a surrogate for elemental carbon in particulate matter
- Extensively used in airborne toxicological studies



Transition Metal

- Iron oxide (Fe_2O_3)
- Represent transition metal oxides in particulate matter
- Water insoluble particle, but soluble in acidic pH



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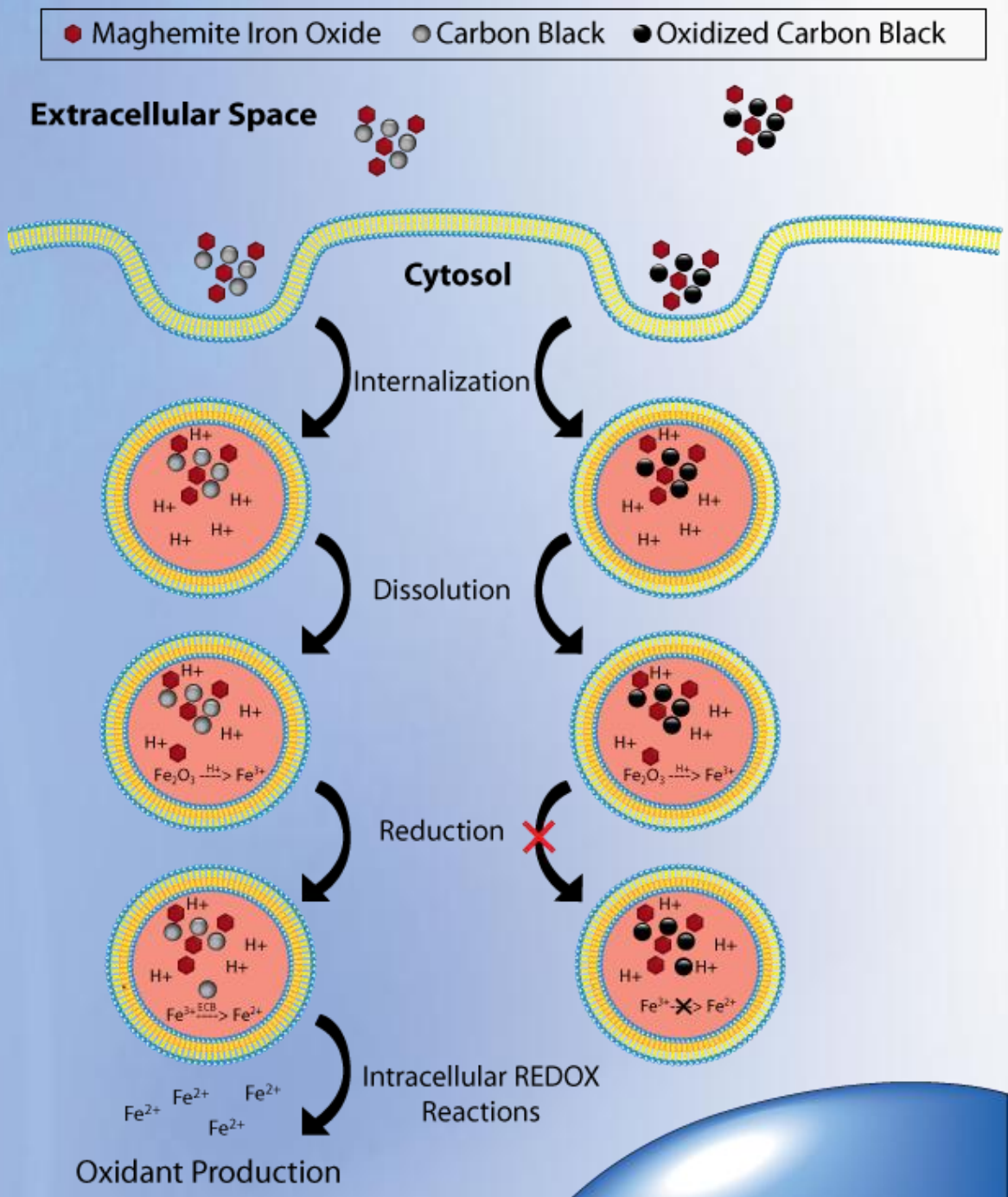
Guo and Sayes, P&FT, 2009

Aim

Determine if Co-exposures to Fe_2O_3 and ECB results in additive or synergistic cytotoxicological effects

Conclusion

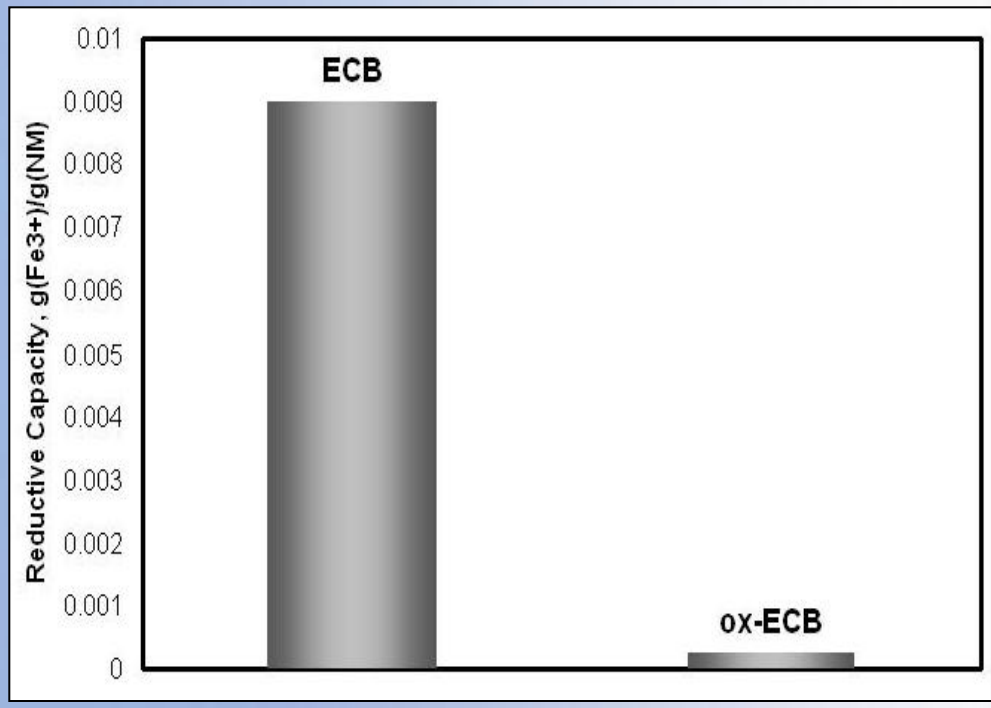
"Co-exposure to carbon black and Fe_2O_3 particles can cause oxidative stress that is significantly greater than the additive effects of exposures to either particle type alone."



Oxidation Decreases Reductive Capacity

Chemically Active Groups on ECB Surface Gives Rise to Surface Redox Capabilities

$$H_2Q_{(s)} + 2Fe^{3+}_{(aq)} \text{------>} \ Q_{(s)} + 2Fe^{2+}_{(aq)} + 2H^+$$



Fenton Reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$$

***Hypothesis*:** Oxidative stress formed in a co-exposure of Fe_2O_3 and ECB can be eliminated by surface oxidation of ECB (termed: ox-ECB).

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Surface Chemistry Analysis with XPS

XPS gives quantitative data on elemental composition and chemistry on the particle surface

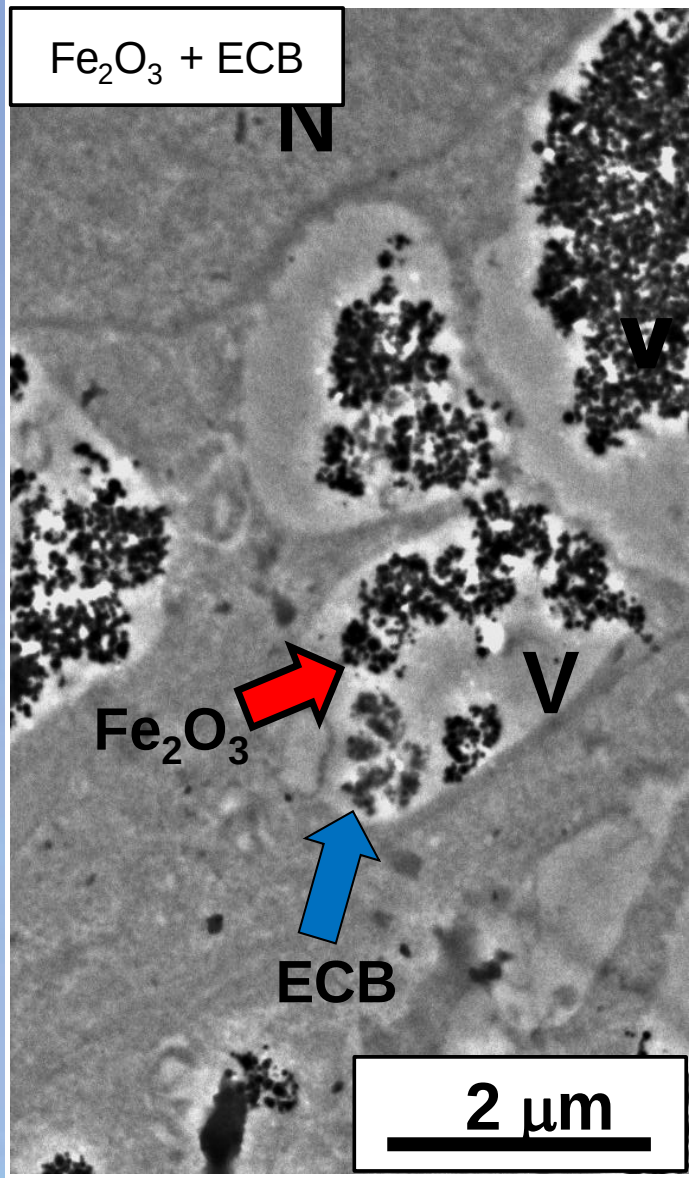
	ECB (%)	Ox-ECB (%)
%C [C/ C+O]	89.88	88.53
%O [O/ C+O]	10.12	11.47
O:C	0.1126	0.1295

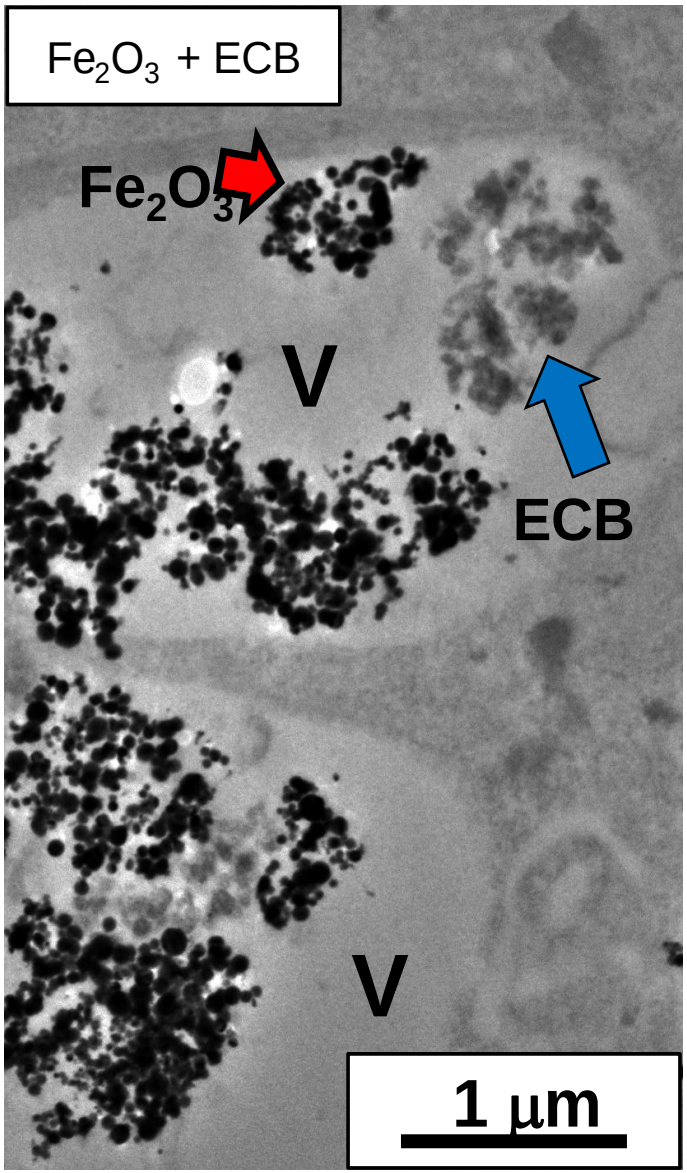
The O:C ratio in ox-ECB is 15% greater than in ECB. This may not seem like a large increase, but edge carbons comprise about 20% of the total carbon content for 50 nm amorphous carbon particles.

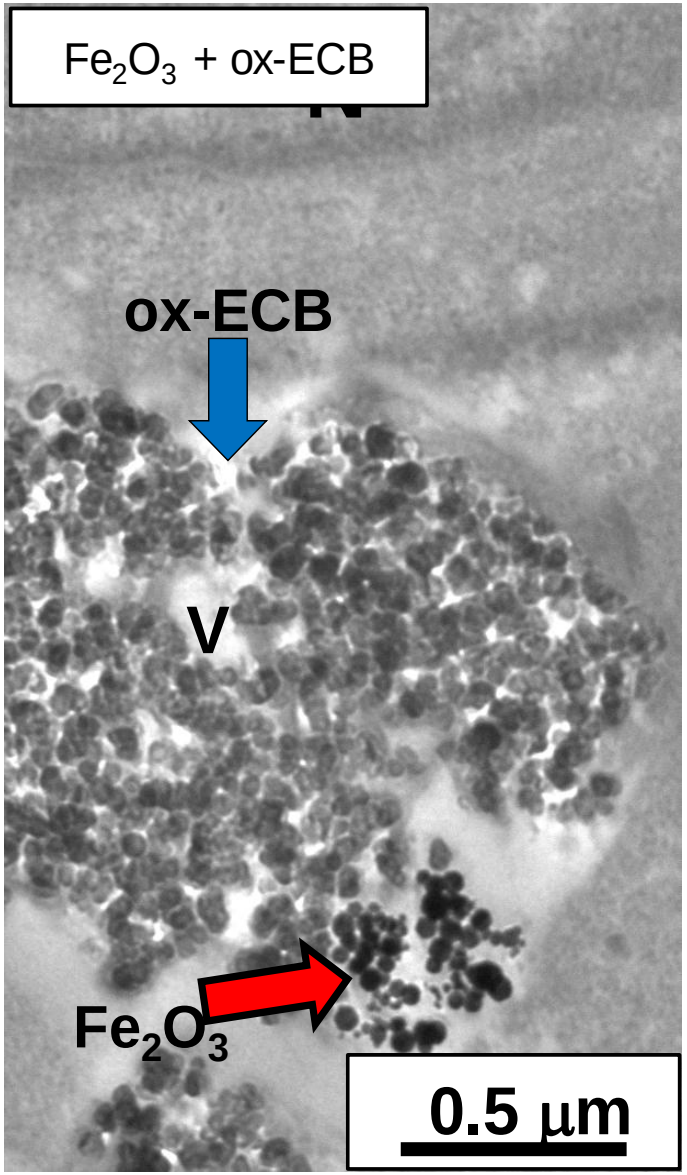
Ratio of $Q_{(s)}$:$H_2Q_{(s)}$ was found to be circa 5000 times greater in ox-ECB than in ECB.

$$H_2Q_{(s)} + 2Fe^{3+}_{(aq)} \not\dashrightarrow Q_{(s)} + 2Fe^{2+}_{(aq)} + 2H^+$$

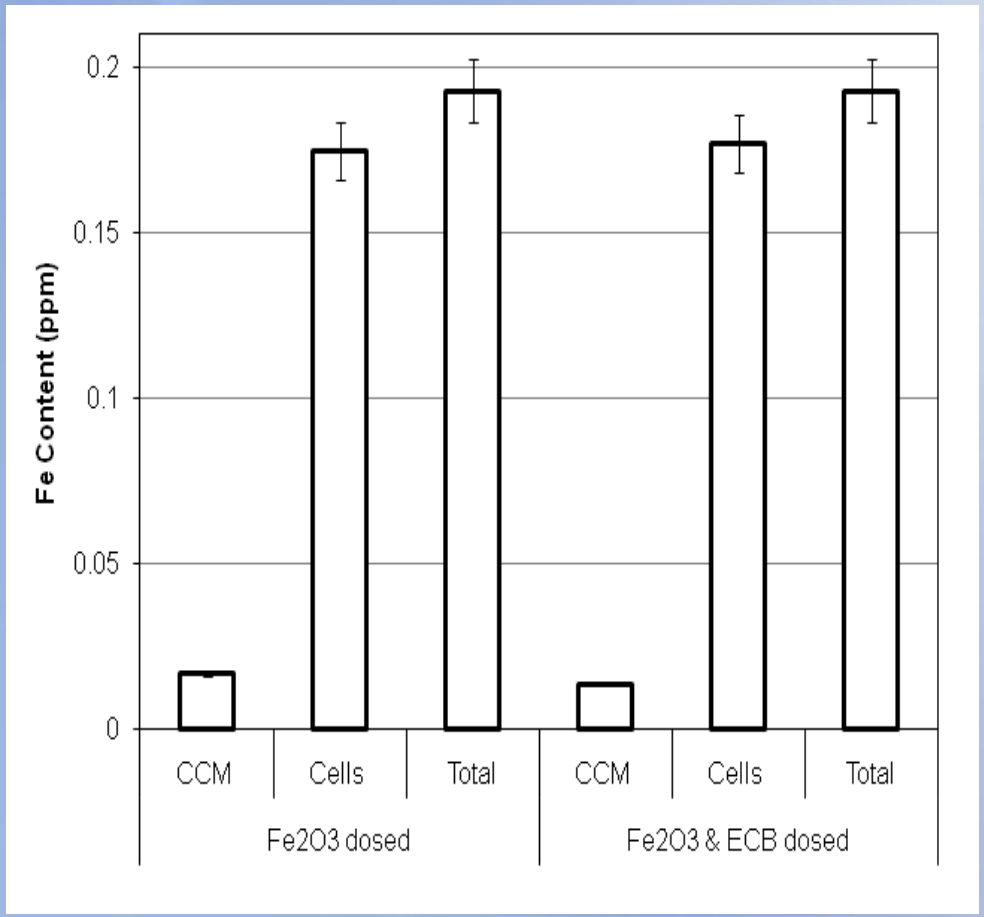
Intracellular Nanoparticle Incorporation





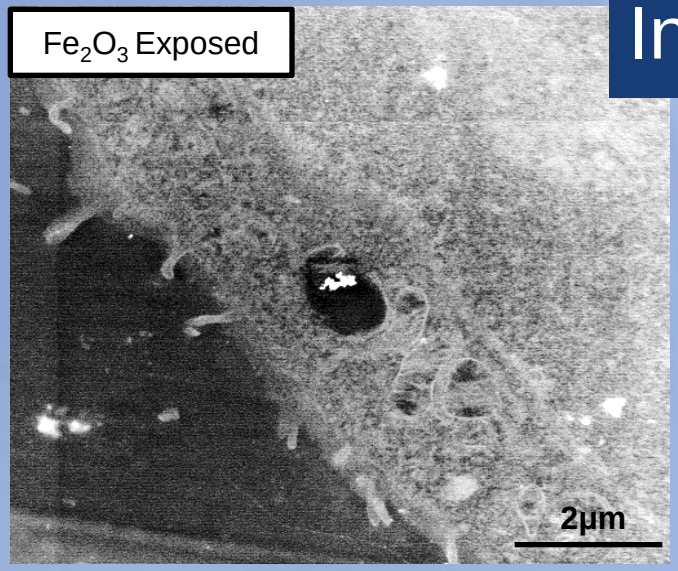


Intracellular Nanoparticle Incorporation

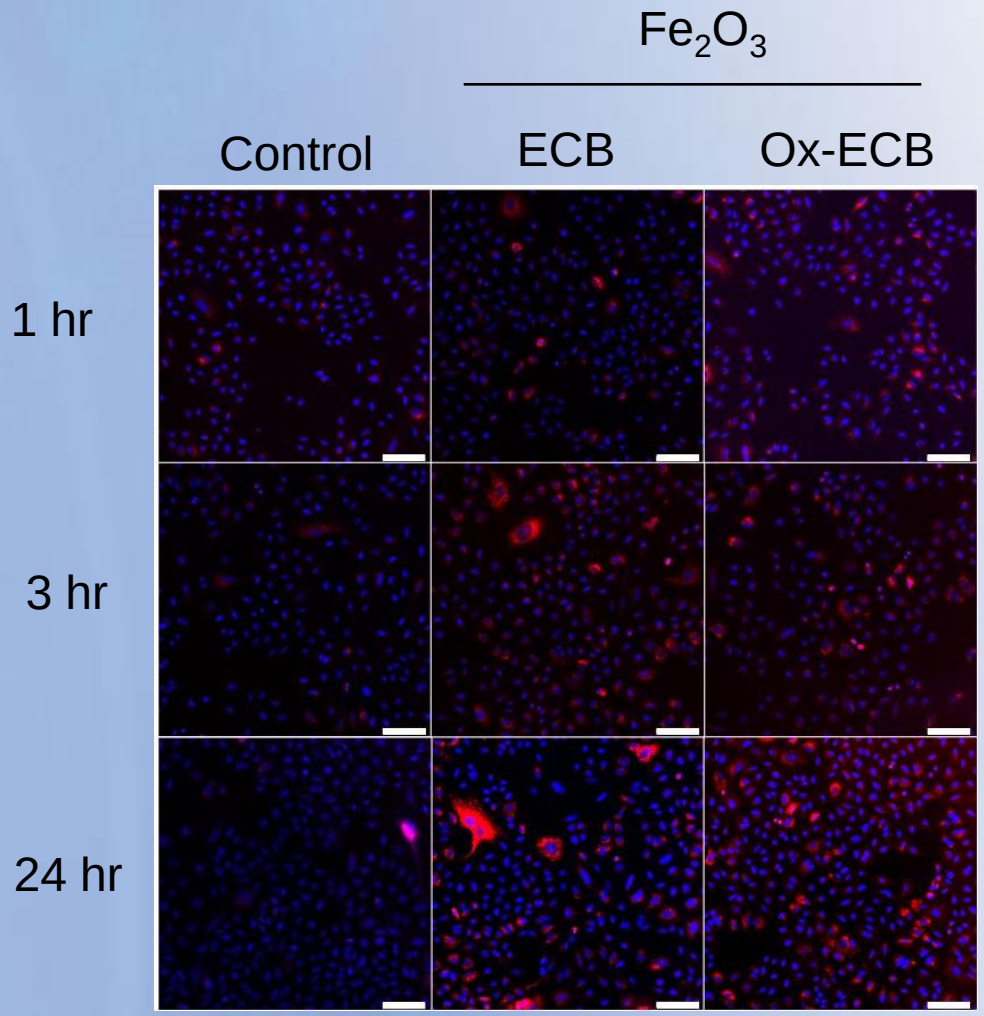


1. A majority of the Fe_2O_3 is internalized into the cells after 24 hours
2. Uptake of Fe_2O_3 is not altered following ECB co-exposure

Intracellular Trafficking of Nanoparticles



STEM-HAADF micrograph of internalized Fe_2O_3 nanoparticle agglomerates



<u>Lysosomal Trafficking</u>

- Nanoparticles accumulate in lysosomes
- pH ≤ 5.2 (pKa Lysotracker Red)
- May alter dynamic nanoparticle properties
- May solubilize water insoluble particles

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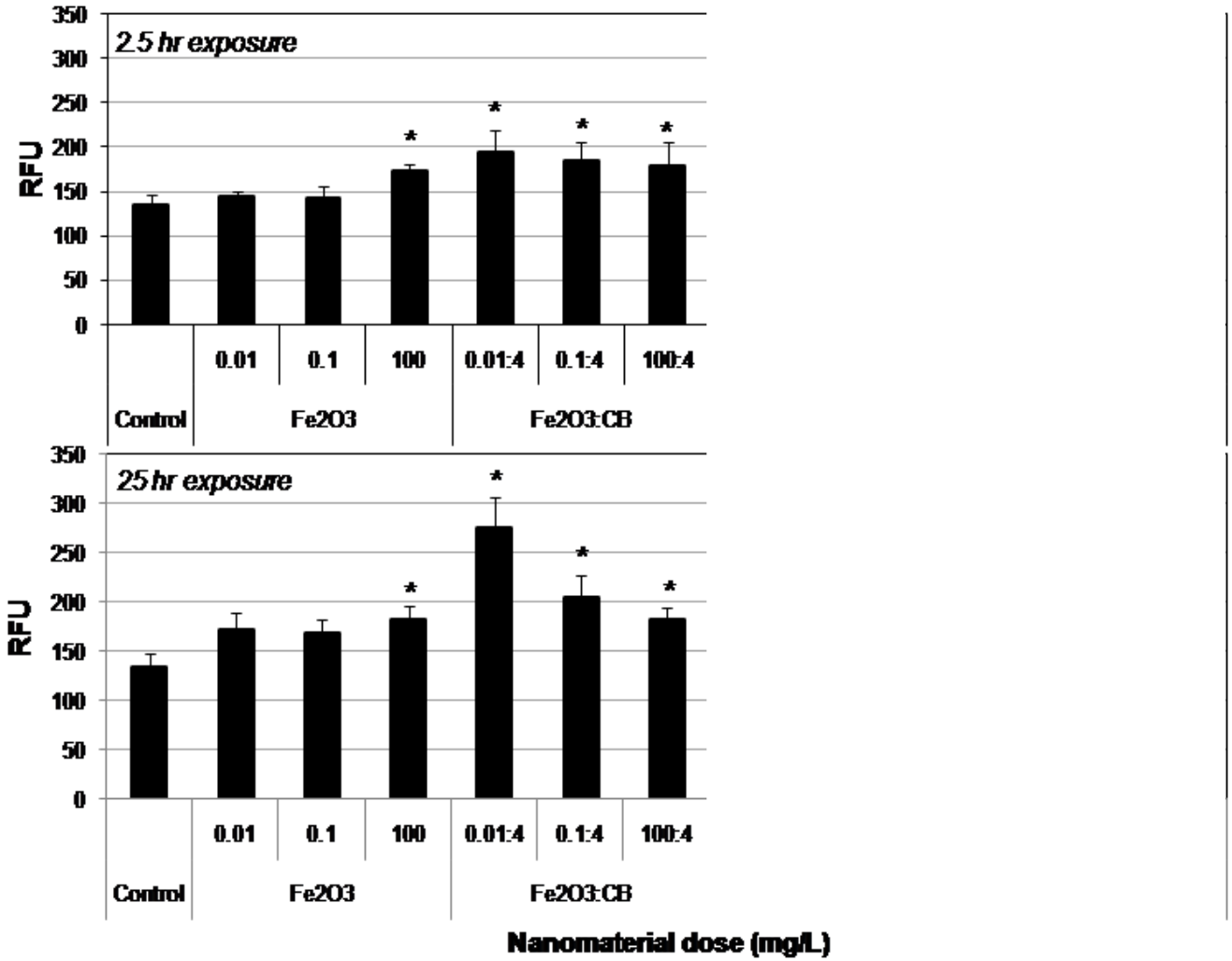
Oxidant Production



* Statistically significant to control population; P<0.05

- Significant increases seen in Fe_2O_3 and ECB co-exposure groups
- Oxidant production eliminated by addition of L-ascorbic acid
- ox-ECB and Fe_2O_3 did not differ from control groups
- Effects greater at 25 hours than at 2.5 hours

Where Do We Go From Here?

Three Key Areas of Growth:

Mathematical/Computational-Based Predictive Models

Alternative Toxicity Testing

Characterization of Real-World Exposure Scenarios

Nanomaterial physicochemical features		Induced toxicological effects		Mathematical techniques		Cross-validation
More fundamental	***More complicated***	***Immediate***	***Systemic***	***Regression Analysis***	***Classification***	
Primary particle size	Zeta potential (as a measure of surface charge)	Cell viability	Metabolism	Linear	Linear Discriminant Analysis	Inter-lab comparisons
Shape	Agglomerate size	Tissue damage	Distribution & accumulation	Non-linear	k-Nearest Neighbor	Beta-testers
Chemical composition	Adsorption of surrounding matrix	Cytokine production	Inflammation	Machine learning	Support Vector Machines	Additional physicochemical data
Specific surface area	Reductive capacity	Membrane damage	Immune response	Causal relationships	Decision Trees or Neural Networks	Additional toxicology data