



Introduction to Nanotoxicology

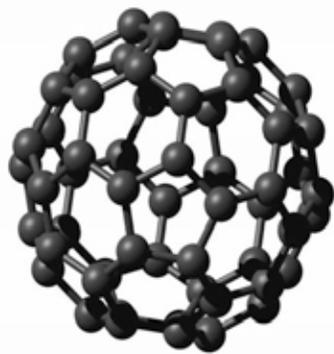
**Center for Occupational and Environmental
Health & The Molecular Foundry**

July 29, 2009

Rick Kelly, MS, CIH

Lawrence Berkeley National Laboratory

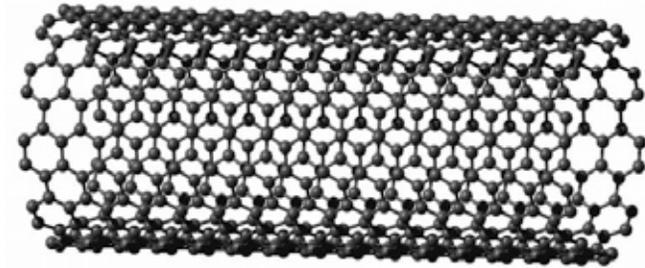
Primarily Talking About *Unbound Engineered Inorganic Nanoparticles*



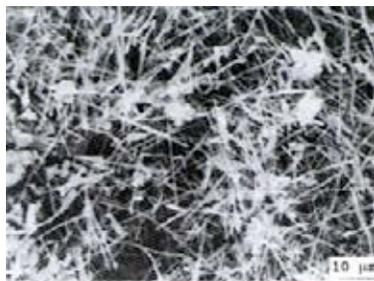
Bucky Ball (C60)



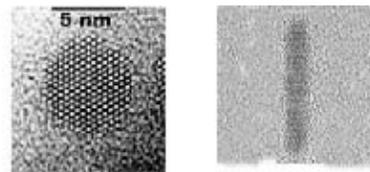
Nanoflowers



Single Wall Carbon Nanotube



Silica Whiskers



Spheres

Rods

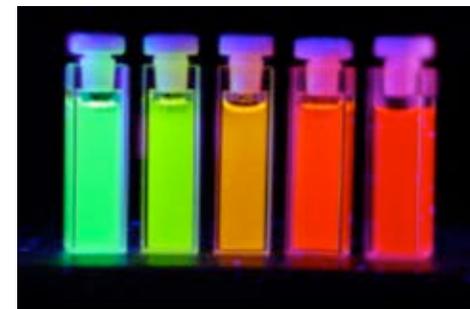


Wires

Ribbons

Tetrapods

Rods, Wires, Shapes

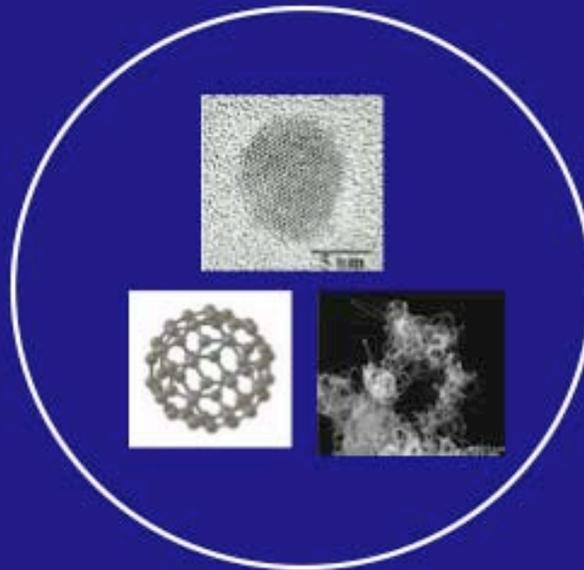


Quantum Nanodots

Broad exposure - many possible routes



End-of-use issues:
Ecological impacts



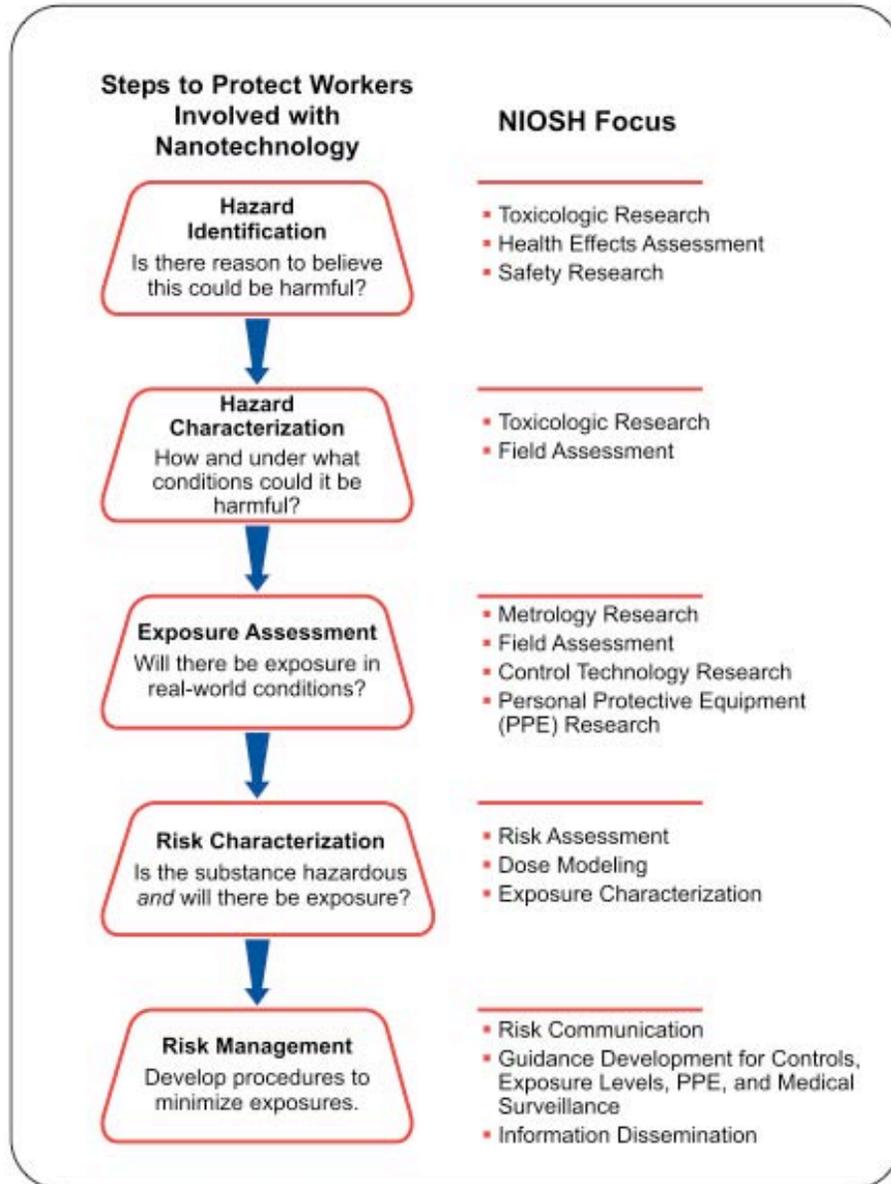
Worker and
laboratory safety

•Also:
Medical
Patients



Direct consumer
contact

The Foundation for Risk Management



- Toxicology is the basis for the rest of the process for protecting people from harmful effects of exposure to engineered nanoparticles
- The *anticipate* in “anticipate, recognize, evaluate and control”

Questions Addressed by Toxicology



- Routes and sites of exposure
- Absorption
- Distribution
- Accumulation
- Metabolism
- Excretion
- Health effects
 - Local
 - Remote
 - Systemic
 - Acute
 - Chronic
 - Heritable
- Shape of dose response curve



Mathieu Joseph Bonaventure
Orfila (1787–1853)

Tools and Mechanisms



- **In vitro**
 - Cell-free preparations
 - Cell cultures
 - Tissue
 - “Tissue surrogates” (complex cell cultures)



In Vitro Limitations



- **Disadvantages**

- May not represent how cells in an animal would really be exposed
- Potentially confounded by model used, exposure procedures
- Doses often very high, physiologically questionable
- Results may not accurately predict health effects in whole animal



In Vivo Animal Studies



- **In vivo animal studies**
 - Acute, sub chronic, chronic
 - Surrogate exposure procedures
 - Injection, intratracheal instillation, aspiration, implantation
 - Real* exposures procedures
 - Ingestion, inhalation, skin contact



In Vivo Limitations



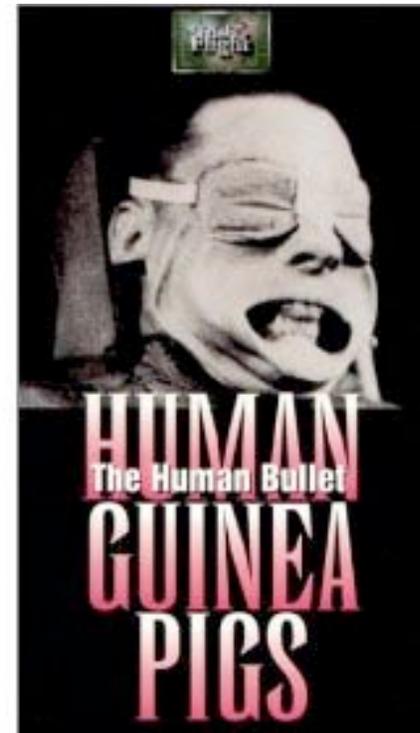
- Rats are not people and may respond differently
 - “Lung Overload” cancer in rats
- Animal tests are cruel



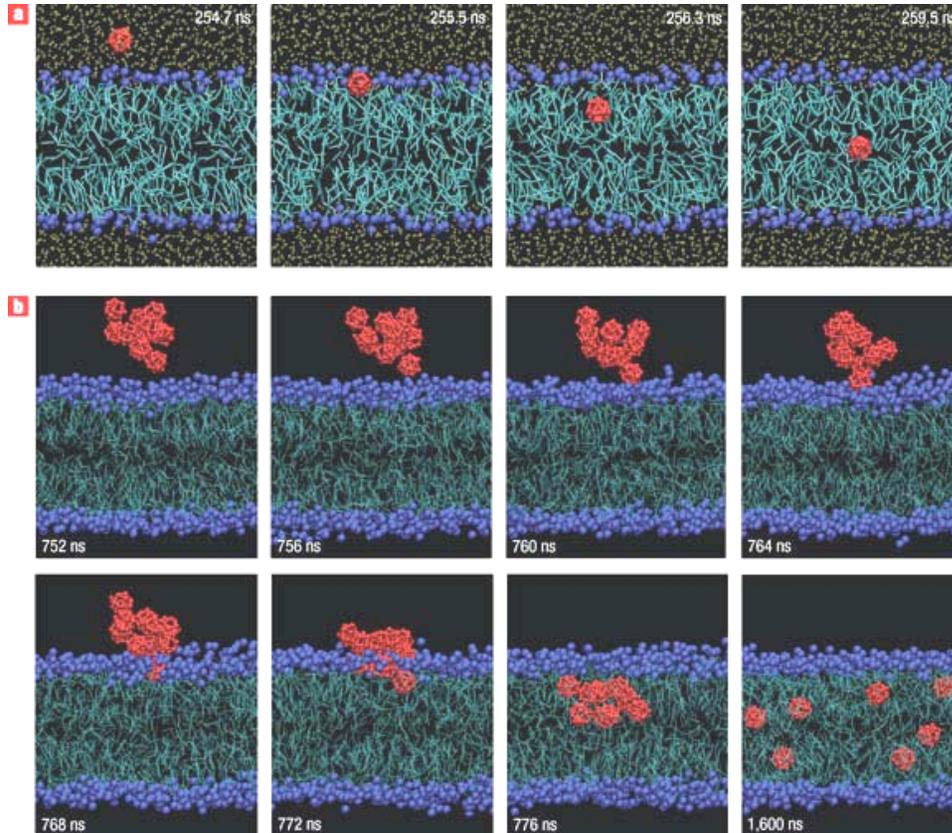
Human Studies



- **Human studies**
 - **Experimental exposures**
 - **Incidental exposures (accidents)**
 - **Epidemiological studies**



In Silico



- **Computer modeling of buckyball translocation through cell membrane**
- **Readily translocates with unknown hazard**

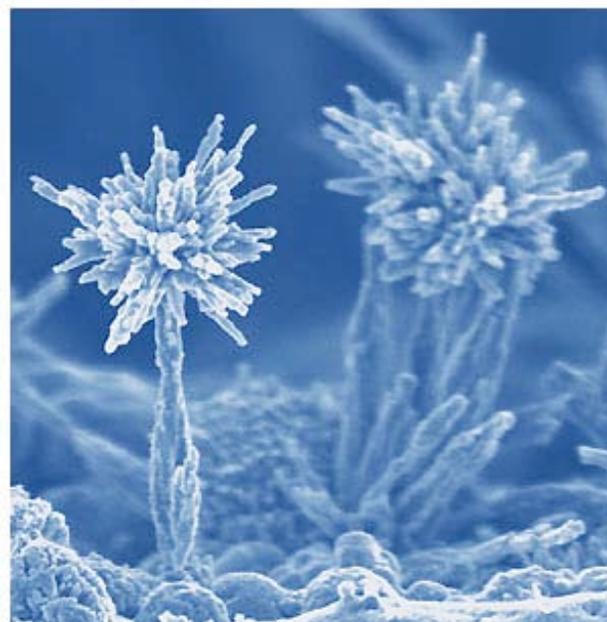


Why Are We Concerned?

Potential for Novel Toxicity



- Properties of nanoscale materials may be fundamentally different from bulk materials of same composition
- Among the new properties of nanoscale materials may be:
 - *Enhanced* toxicity of toxic materials
 - *New* toxicological properties not seen in bulk material



Established Example of Particle Size Dependent Toxicity

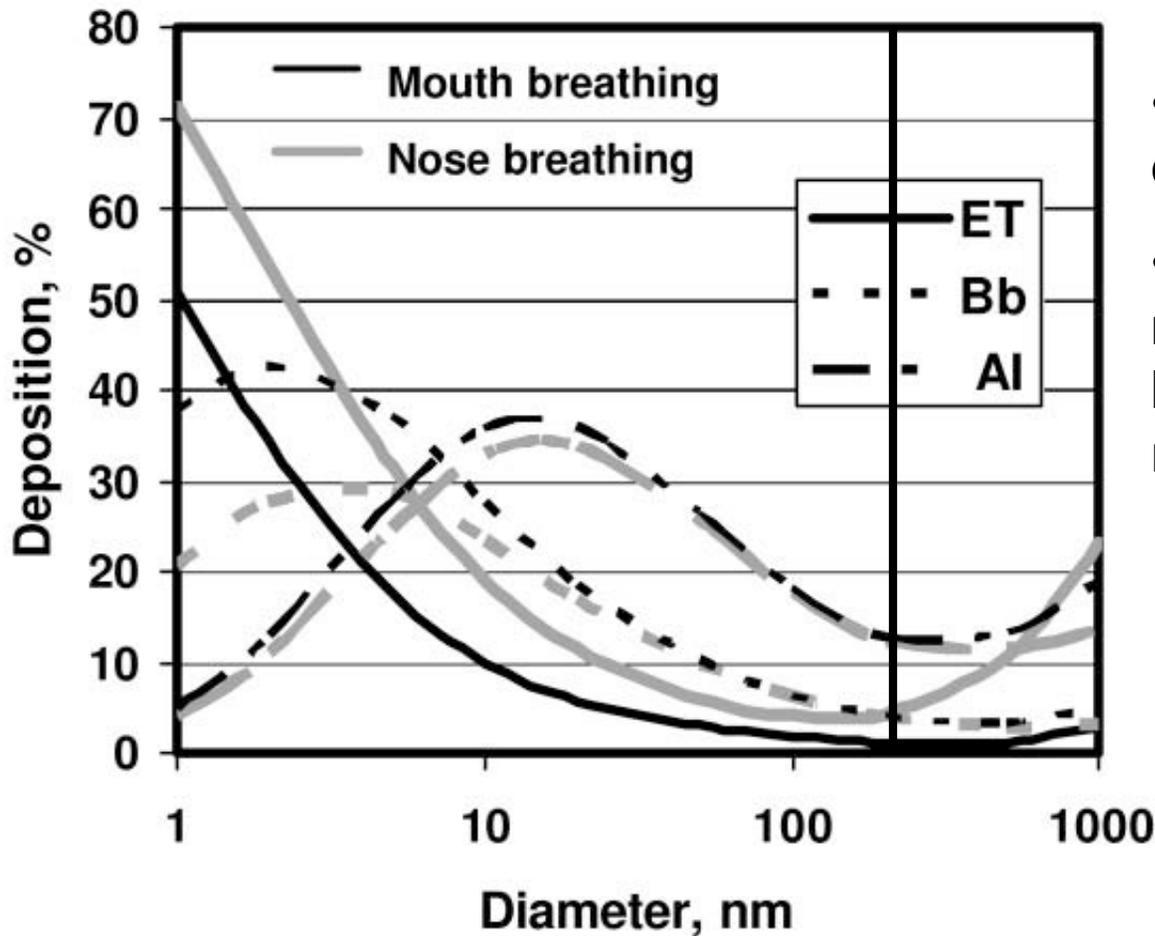


- **Free crystalline silica**
 - **Most toxic when $<10\ \mu\text{m}$, for two reasons**
 - **Alveolar deposition of micrometer range particles**
 - **Increased surface area causes oxidative stress via catalysis**
 - **“In conclusion, our data show that quartz elicits DNA damage in rat and human alveolar epithelial cells and indicate that these effects are driven by hydroxyl radical-generating properties of the particles -- Schins et al, 2002.”**
- **Where in the respiratory tract do nanoparticles deposit?**



http://www4.umdj.edu/cswaweb/rad_teach/silicosis.html

Respiratory Tract Deposition



- Mostly alveolar down to ~10 nm
- Mostly upper respiratory (nose) and bronchial below ~10 nm!

Borm et al. 2006,
based on ICRP 66

Ambient Fines and Ultrafines Are Associated With Disease

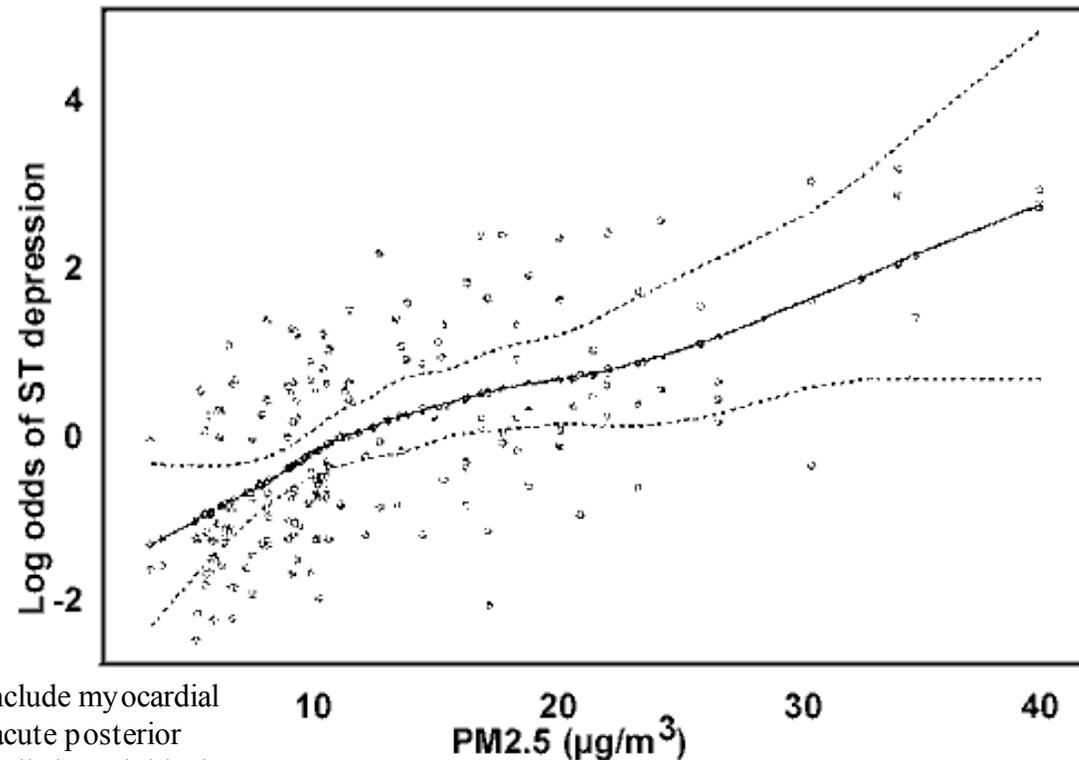


- **Ambient fines and ultrafines are associated with increased cardiovascular and respiratory events, including death, in susceptible populations**

Particulate Air Pollution and Risk of ST-Segment Depression During Repeated Submaximal Exercise Tests Among Subjects With Coronary Heart Disease

Juha Pekkanen et al 2002

Reflects myocardial ischemia

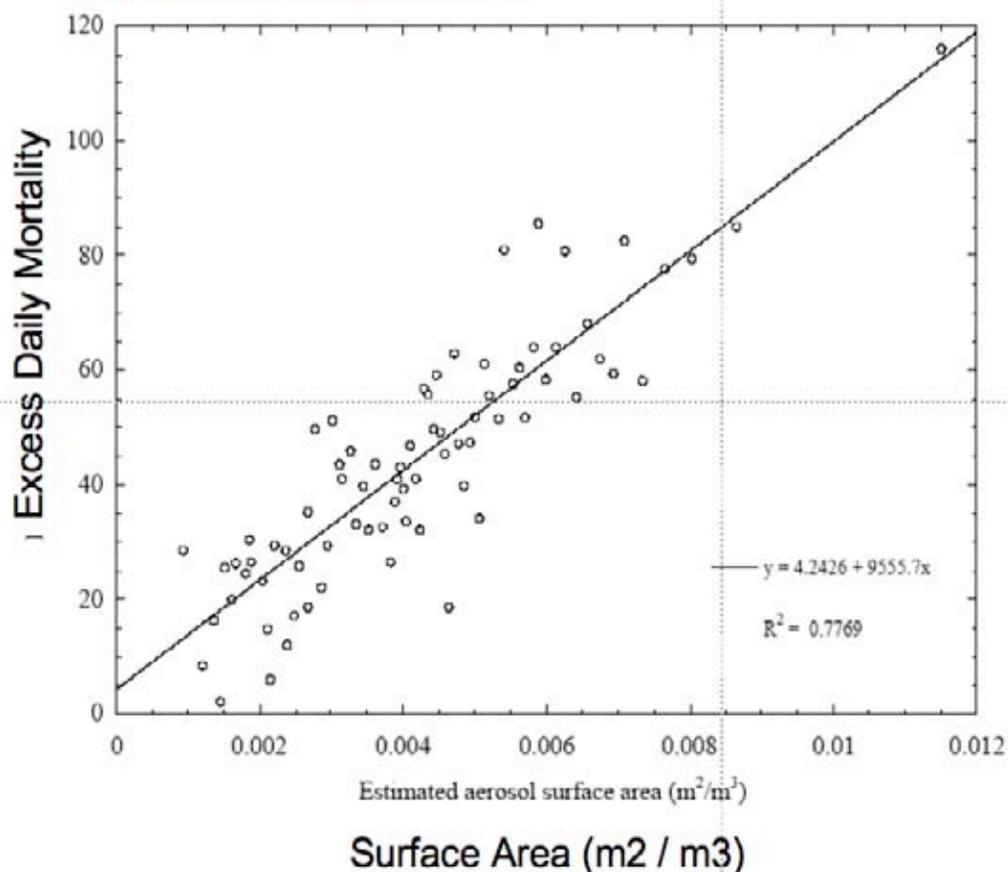


Causes of ST segment depression include myocardial ischemia, digoxin effect, ventricular hypertrophy, acute posterior myocardial infarction, pulmonary embolus, left bundle branch block

Attack of the Killer London Fog

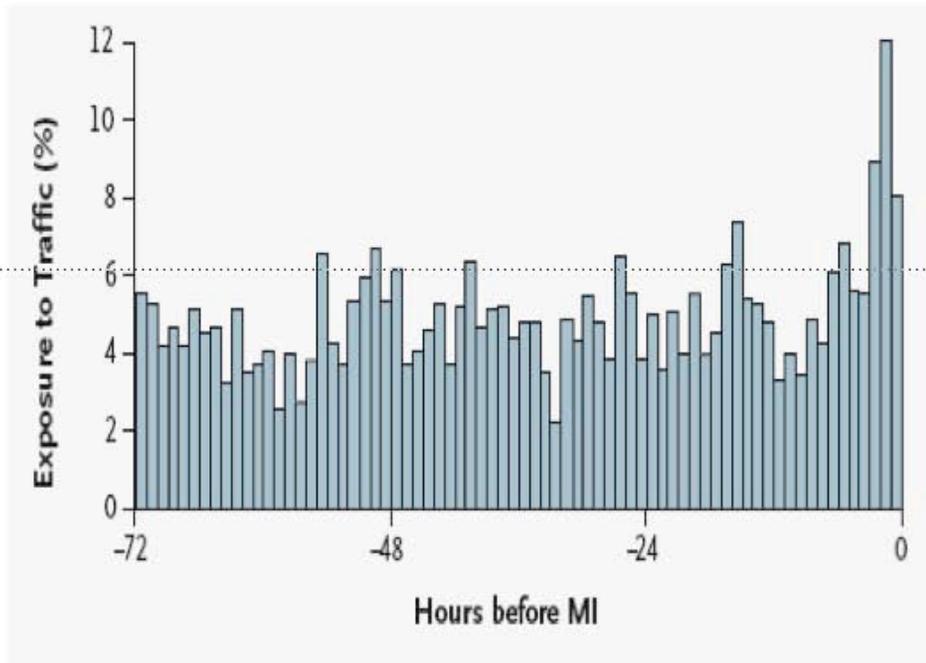


Mortality Vs. Surface Area Concentration



Ultrafine Exposure Effects Heart

Onset of myocardial infarction (MI) associated with exposure to traffic



Peters et al., 2004 NEJM

- Increased coagulability of blood Ruckerl et al. (2005)
- Reduced heart-rate variability Liao et al. (1999)
- Increased likelihood of cardiac arrhythmia Peters et al. (2000)



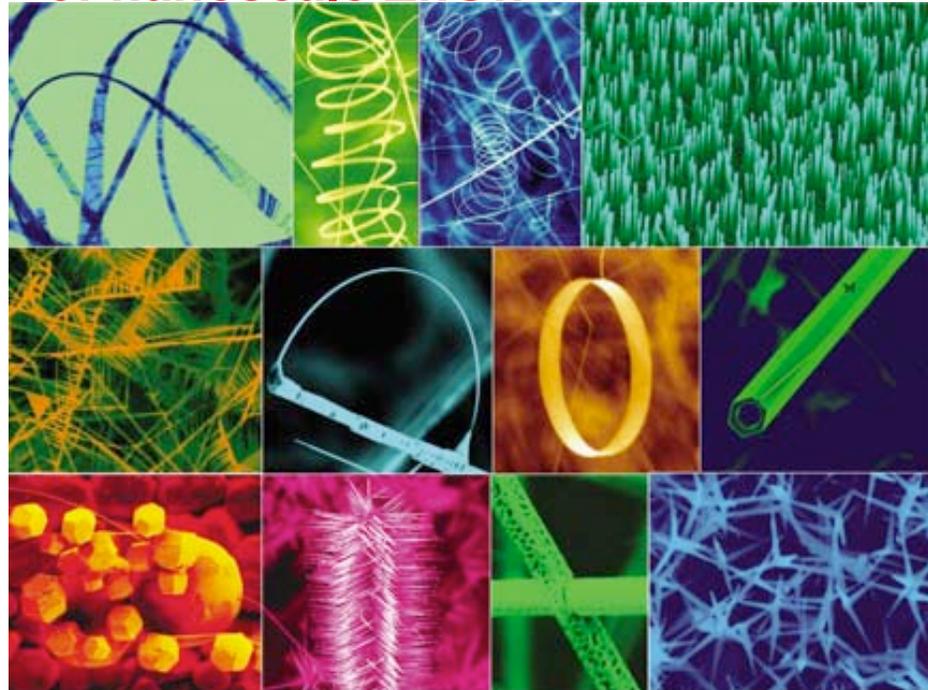
It is not going to be easy to sort out



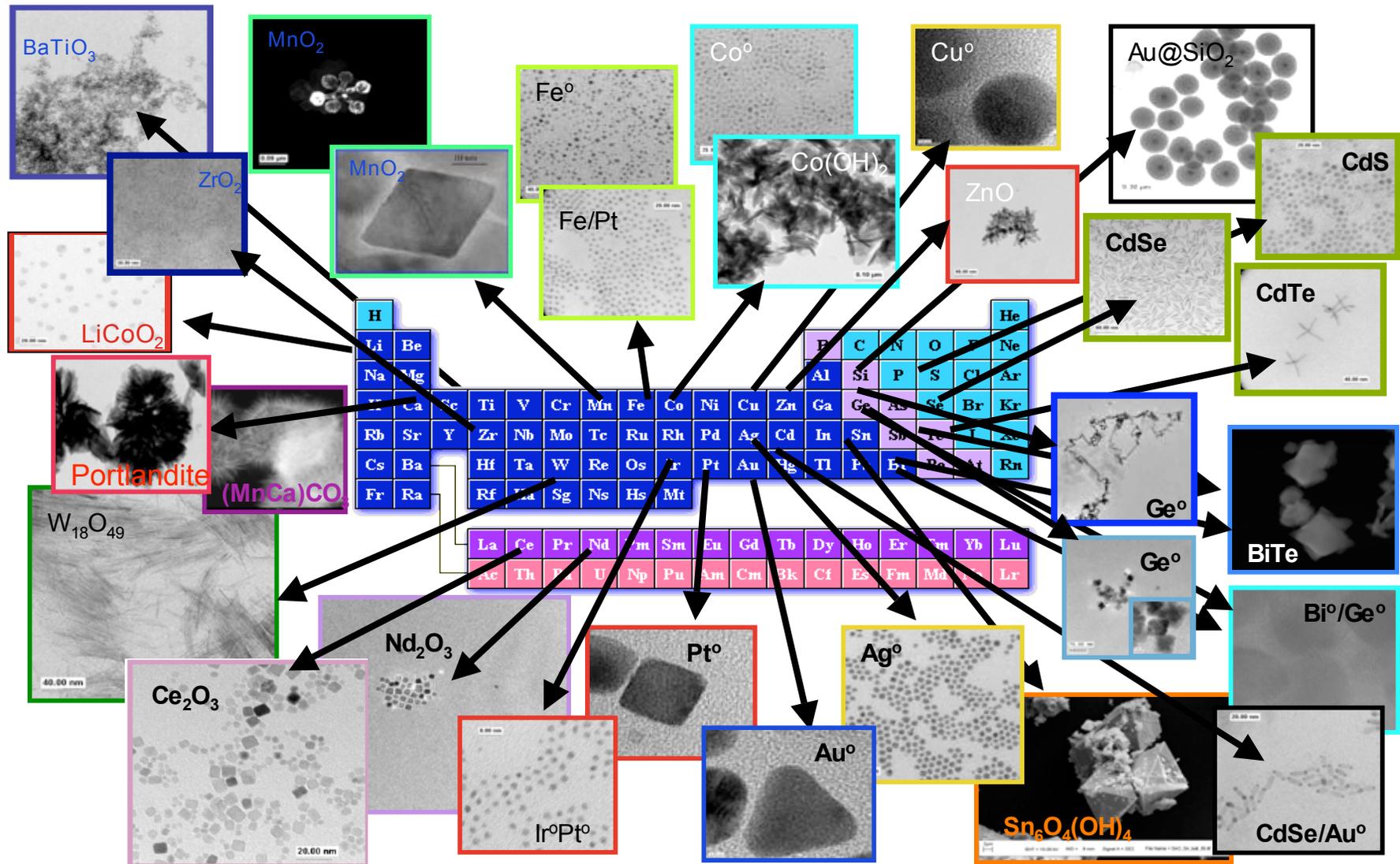
- **Many variables may effect toxicity**

- **Size**
- **Shape**
- **Chemistry**
- **Crystal structure**
- **Water solubility**
- **Surface area**
- **Surface coating**
- **Agglomeration state**
- **Density**
- **Dispersability**
- **Porosity**
- **Surface charge**
- **Conductivity**
- **Contaminants**
- **Manufacturing method**

One chemistry but many forms of nanoscale ZnO!!



Extremely Broad Chemistries



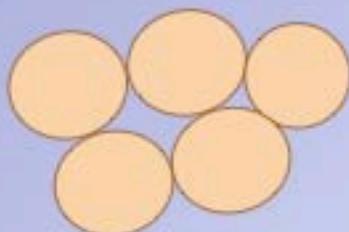
This from one set of labs at the University of New Mexico

Nanoparticle Physicochemical Properties: What is Important?

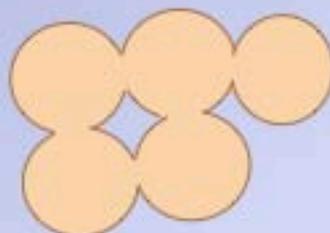
Primary vs. Agglomerated vs. Aggregated Nanoparticles



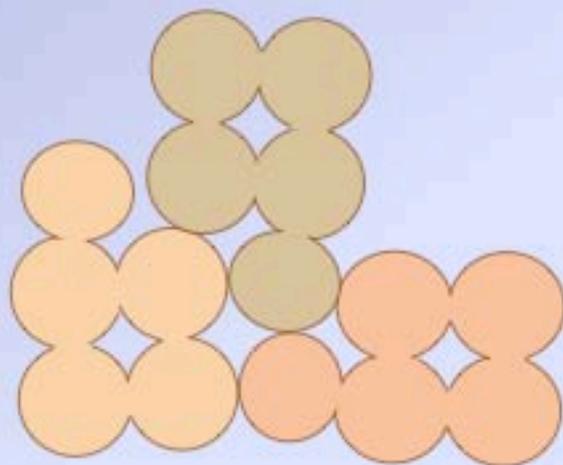
Primary Particle
e.g. quantum dot



Agglomerated Primary Particles (Agglomerates)
e.g. nC_{60} fullerenes



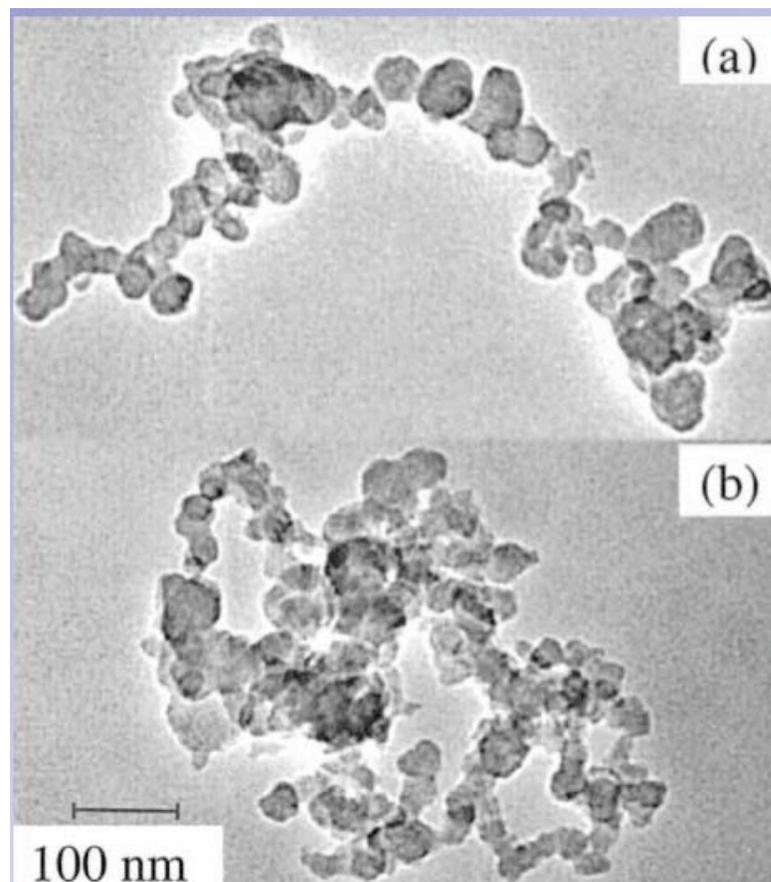
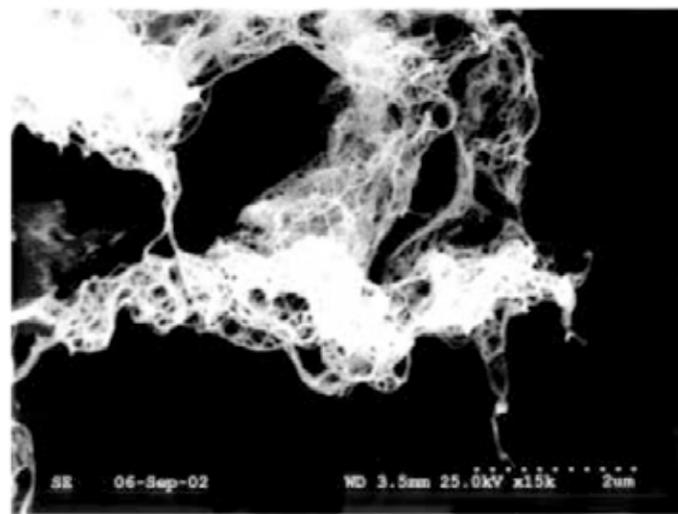
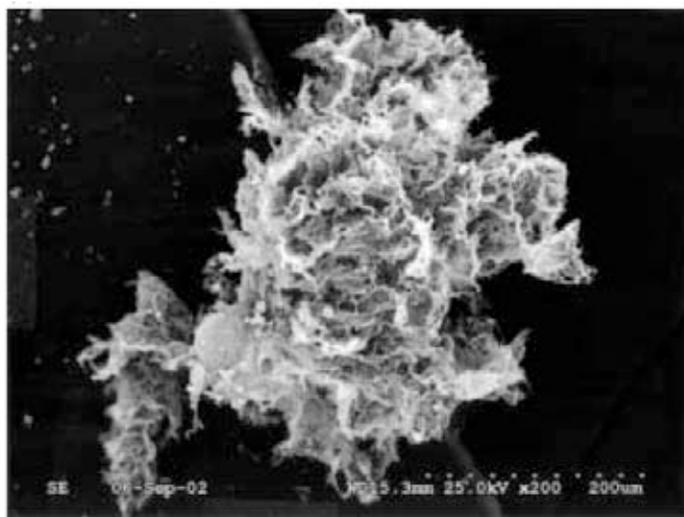
Aggregated Primary Particles (Aggregates)
e.g. carbon black



Agglomerated Aggregates
e.g. ambient air PM

From Alison Elder, U
Rochester

Nanoparticle Agglomeration is the Norm

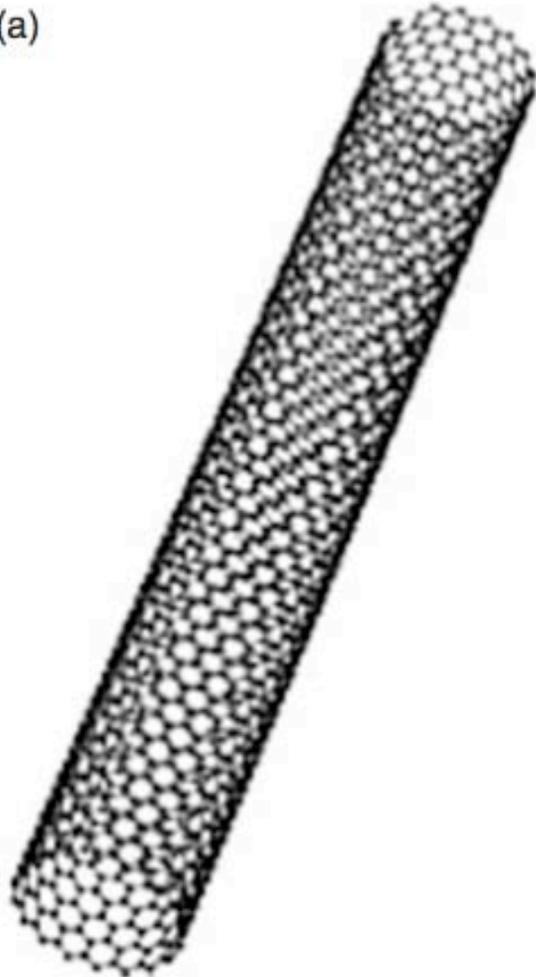


from: Xiong & Friedlander, 2001; Roth et al., 2004; Teng et al., 2003

Contamination of Materials Leads to False Conclusions

Nanotechnology

(a)



(b)

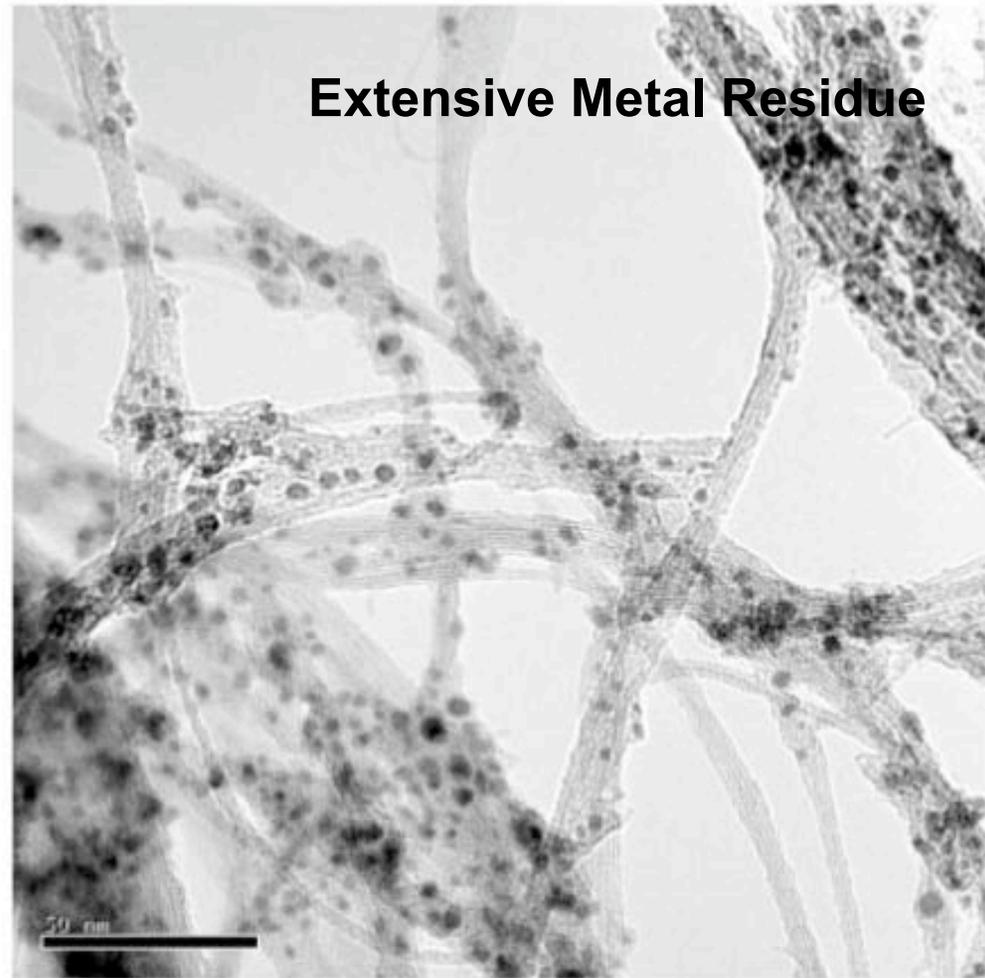
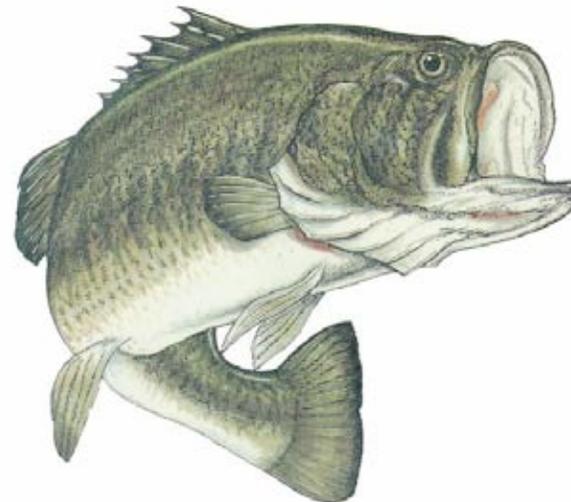
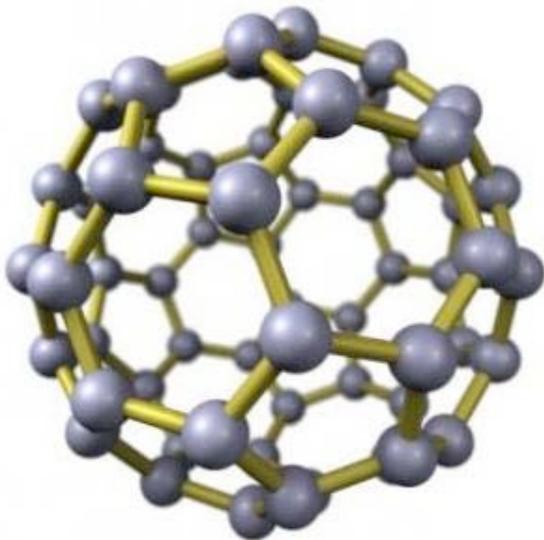


Fig. 1. Single-walled carbon nanotubes. (a) Schematic diagram of a single-walled carbon nanotube © Chris Ewels. (b) Transmission electron micrograph of as-produced single-walled carbon nanotube, showing aligned clusters of nanotubes (nanoropes) and nanometre-diameter metal catalyst particles, used in the production process.

More Material Contamination



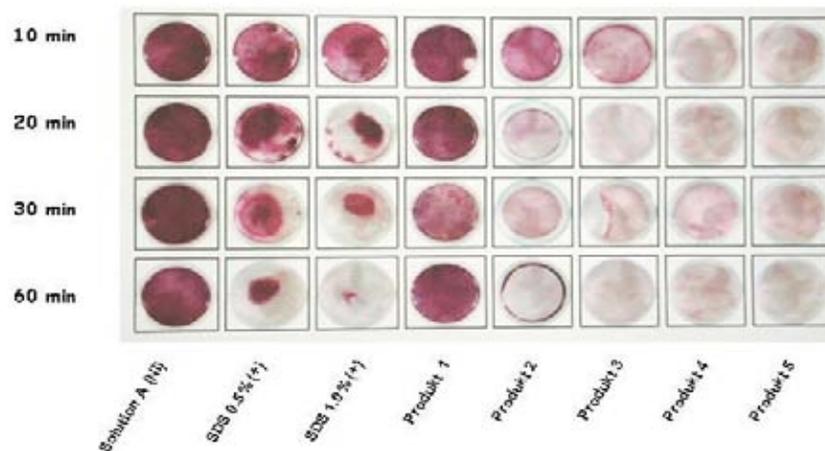
- Oberdorster (2004) found high Buckyball neurotoxicity to large mouth bass when present in the water at only 0.5 ppm
- Apparently confounded by residual THF solvent used in manufacture the buckyballs



Uses of Tests Not Compatible with Nanoparticles



- MTT test--measures mitochondrial toxicity
- Lack of red indicates inactive mitochondria
- Early studies said carbon nanotubes showed high toxicity in this test
- In fact, CNTs interfere with this assay and make it almost use



This Seems to be a Common Problem with Nanoparticles



□ 1: [Toxicol Appl Pharmacol](#). 2009 Jan 15;234(2):222-35. Epub 2008 Oct 17.

Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line.

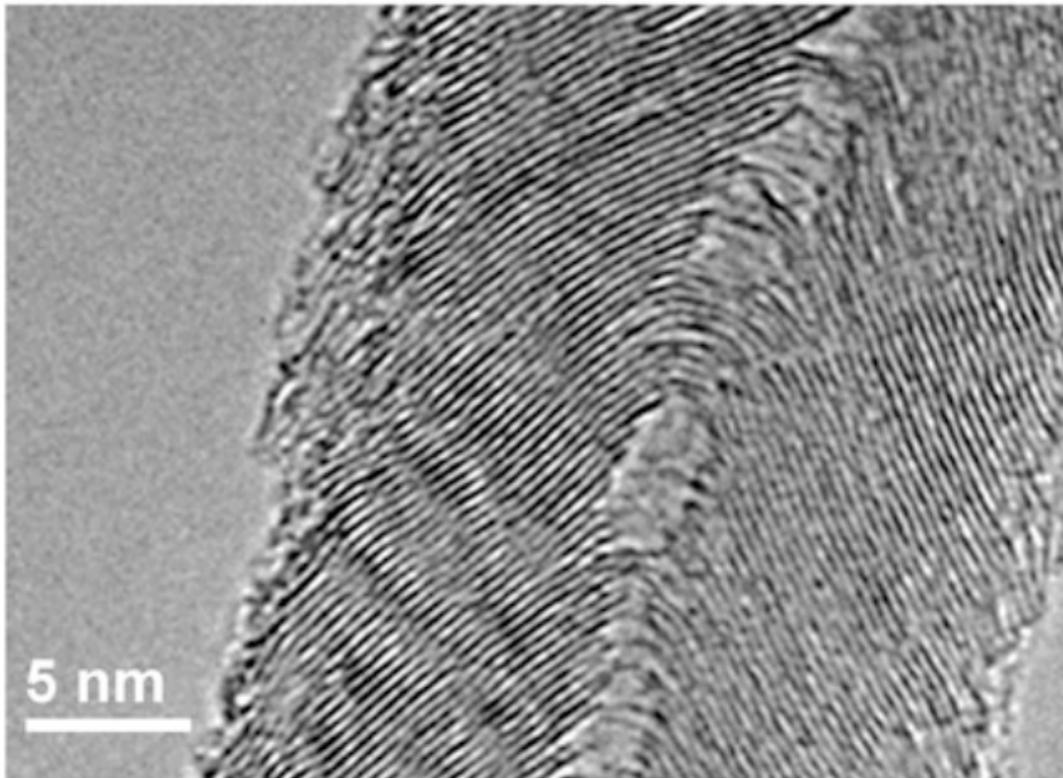
[Monteiro-Riviere NA](#), [Inman AO](#), [Zhang LW](#).

Center for Chemical Toxicology Research and Pharmacokinetics, North Carolina State University, 4700 Hillsborough Street, Raleigh, NC 27606, USA.
Nancy_Monteiro@ncsu.edu

Single-walled carbon nanotubes (SWCNT), fullerenes (C(60)), carbon black (CB), nC(60), and quantum dots (QD) have been studied in vitro to determine their toxicity in a number of cell types. Here, we report that classical dye-based assays such as MTT and neutral red (NR) that determine cell viability produce invalid results with some NM (nanomaterials) due to NM/dye interactions and/or NM adsorption of the dye/dye products. In this study, human epidermal keratinocytes (HEK) were exposed in vitro to CB, SWCNT, C(60), nC(60), and QD to assess viability with calcein AM (CAM), Live/Dead (LD), NR, MTT, Celltiter 96 Aqueous One (96 AQ), alamar Blue (aB), Celltiter-Blue (CTB), CytoTox Onetrade mark (CTO), and flow cytometry. In addition, trypan blue (TB) was quantitated by light microscopy. Assay linearity (R(2) value) was determined with HEK plated at concentrations from 0 to 25,000 cells per well in 96-well plates. HEK were treated with serial dilutions of each NM for 24 h and assessed with each of the viability assays. TB, CAM and LD assays, which depend on direct staining of living and/or dead cells, were difficult to interpret due to physical interference of the NM with cells. Results of the dye-based assays varied a great deal, depending on the interactions of the dye/dye product with the carbon nanomaterials (CNM). Results show the optimal high throughput assay for use with carbon and noncarbon NM was 96 AQ. This study shows that, unlike small molecules, CNM interact with assay markers to cause variable results with classical toxicology assays and may not be suitable for assessing nanoparticle cytotoxicity. Therefore, more than one assay may be required when determining nanoparticle toxicity for risk assessment.

PMID: 18983864 [PubMed - indexed for MEDLINE]

Nanotube, NOT!!



- **Representative “carbon nanotube” from Mitchell et al (2007) inhalation study is in fact a nanofiber.**
- **Cheap Tubes!**
- **The authors didn’t know the difference!**

Really Cheap Tubes!



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Translocation to Blood from Lung, NOT



- **Example 2: Airborne exposure study did not account for grooming and resulting GI exposure, **overestimated translocation from lung from inhaled particles****



Problems With Surrogate Dosing Models



- **Artificial dosing methods may cause epiphenomenal effects**
 - **Right—Animals choked to death by CNTs instilled in their lungs**

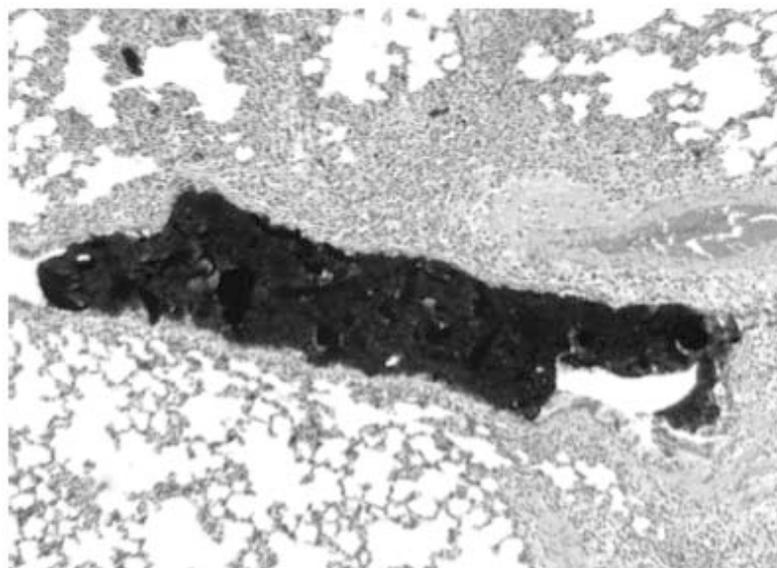
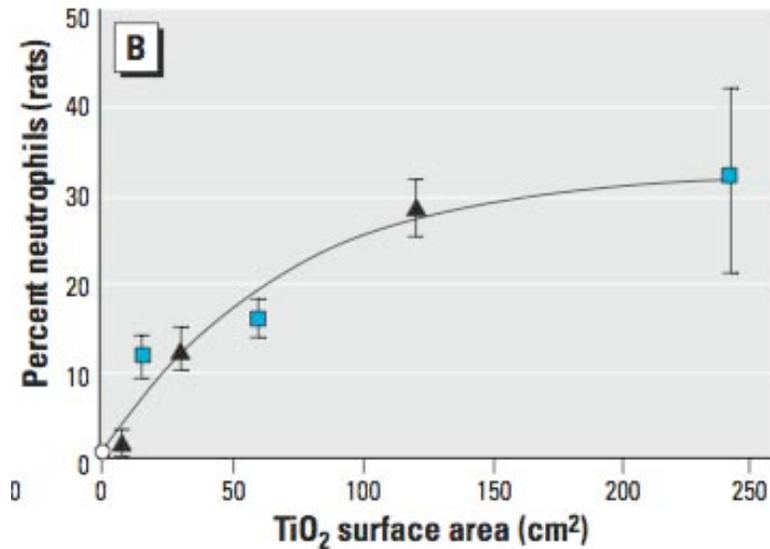
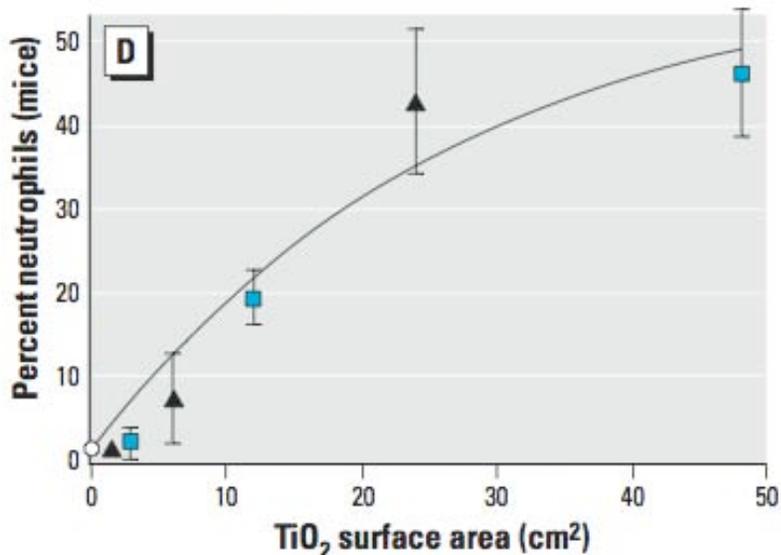


Figure 1. Light micrograph of lung tissue from a rat exposed to 5 mg/kg SWCNT (a few hours after exposure). The major airways are mechanically blocked by the SWCNT instillate. This led to suffocation in 15% of the CNT-exposed rats and was not evidence of pulmonary toxicity of SWCNT.

Interspecies Variability



- TiO₂ more toxic to mice than rats
- TiO₂ dust overload causes cancer in rats but not other species



Mechanisms of Toxicity



Drivers of Toxicity

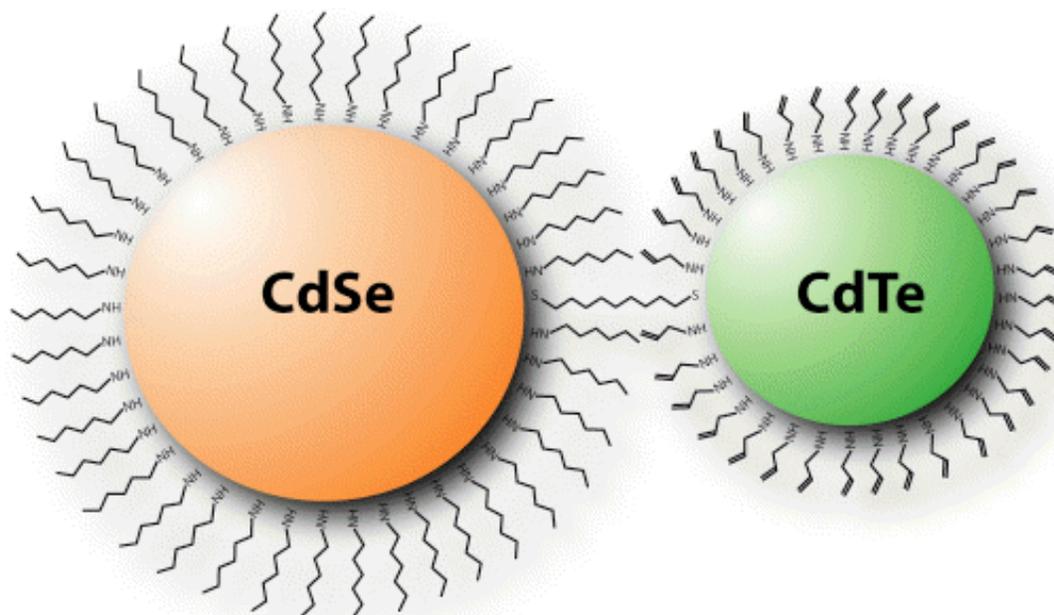


- **Intrinsic elemental (chemical) toxicity**
 - Usually soluble materials, individual atoms or ions interfere with biological systems
 - Lead, cadmium, benzene, fluoride, etc
 - Usual dose metric is mass
- **Morphology-driven toxicity**
 - Fiber toxicity
 - Asbestos, fibrous zeolites, MMMF
 - Usual dose metric is fiber count
- **Surface reactivity driven toxicity**
 - Surface catalyzes damaging reactions
 - Surface area is likely the most relevant dose metric

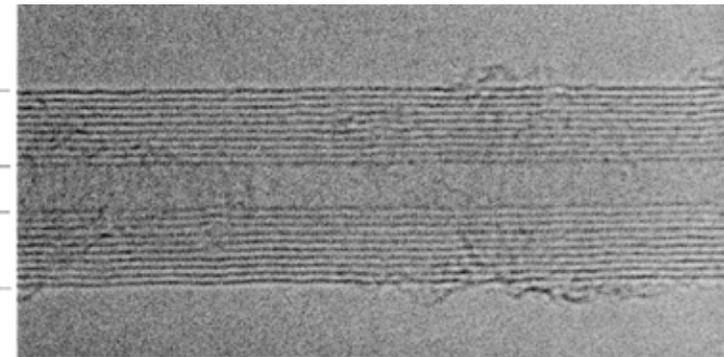
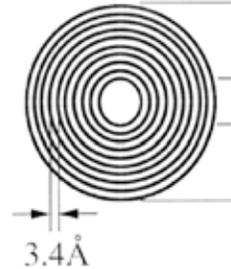
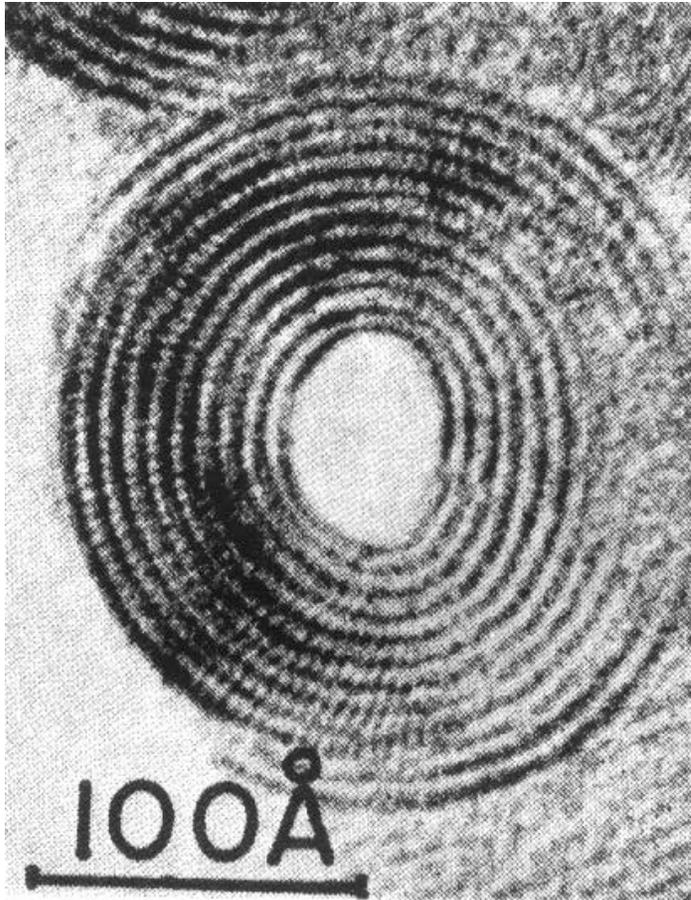
Chemistry Driven Toxicity



- **Semiconductor nanocrystals, quantum dots**
 - Se, Cd, Pb, Te, Zn, S, Ga, Sb, As, In
 - Elemental toxicity (ions) *partially* explains quantum dot toxicity**



Morphology Driven Toxicity



Two similar appearing *nanotubes*

- Chrysotile asbestos (left)
- Multiwall carbon nanotube (above)

Similar toxicity?

www.gly.uga.edu/schroeder/geol6550/CM07.html

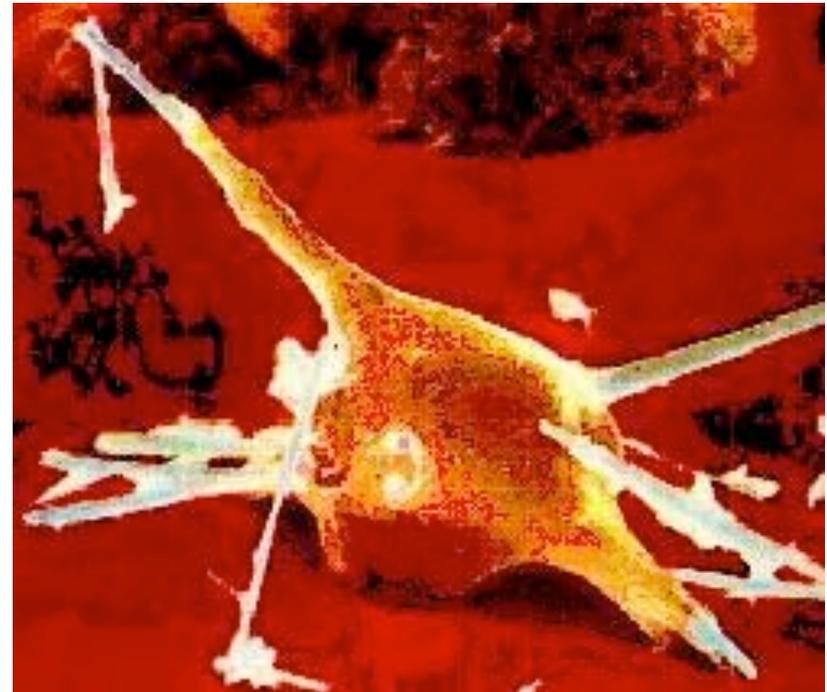
Morphology-Driven Toxicology

Fiber Toxicology



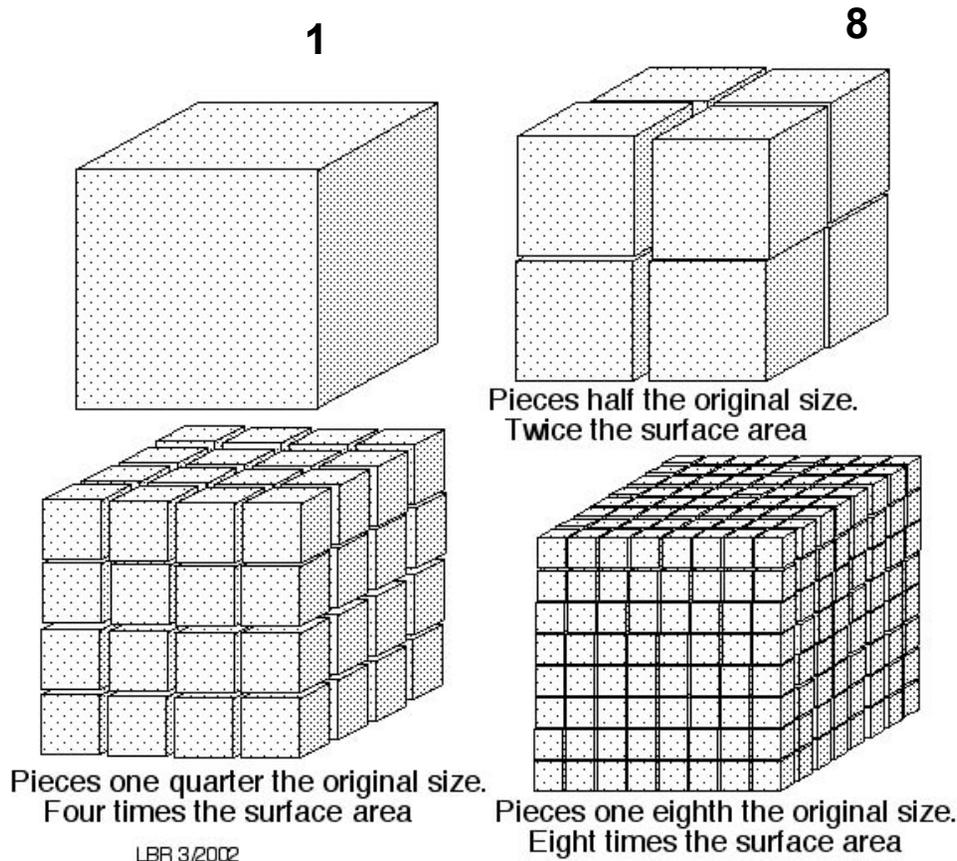
- **Key factors contributing to toxicity:**

- Diameter < 1000 nm
- Length >5,000 nm:
- High biopersistence
- Poor pulmonary clearance

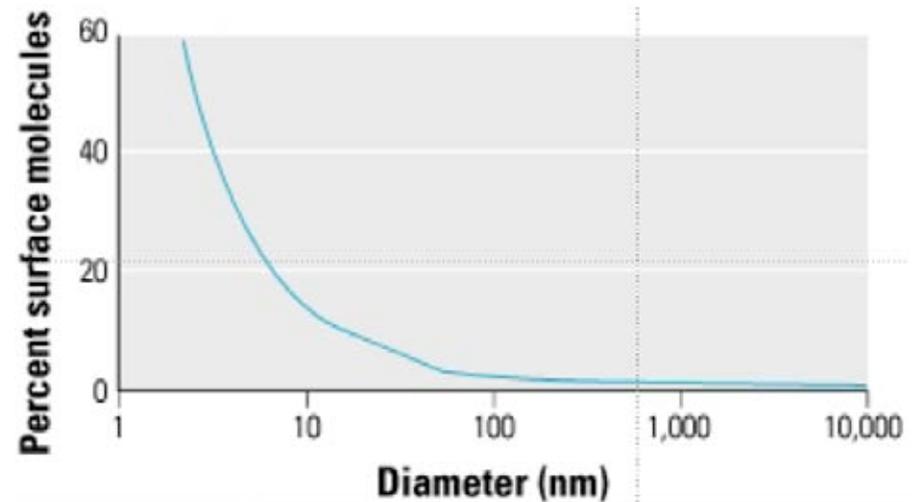


- **More on carbon nanotubes to come**

Surface Area Driven Toxicity



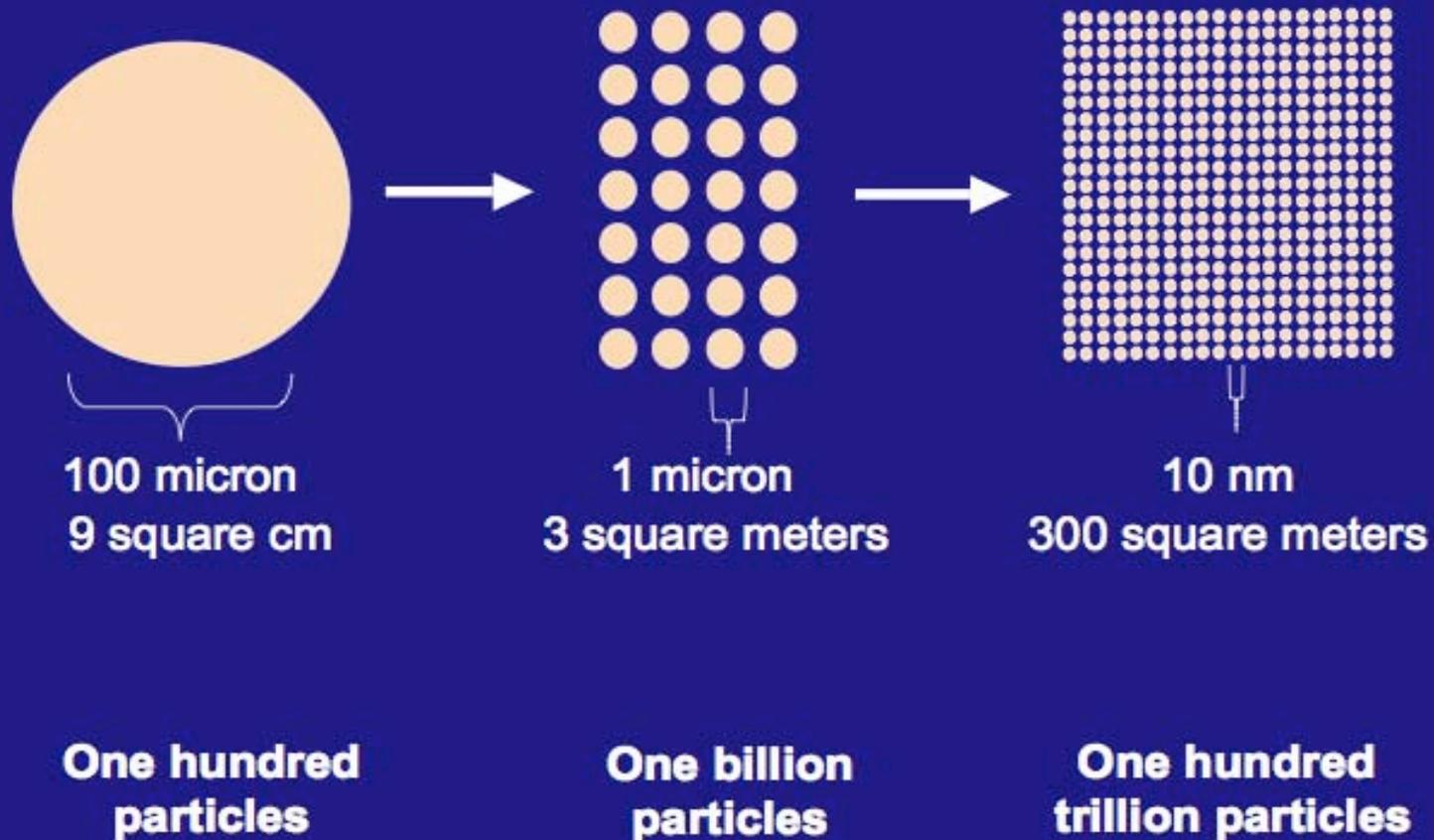
- Nanoparticle surface area is huge
- More surface area = more catalysis
- Approaches 100% of atoms on the surface



•www.gly.uga.edu/railsback/1121WeatheringArea.jpeg

What's the right dose measure?

One milligrams of quartz sand:



Surface Area May Be Critical Metric

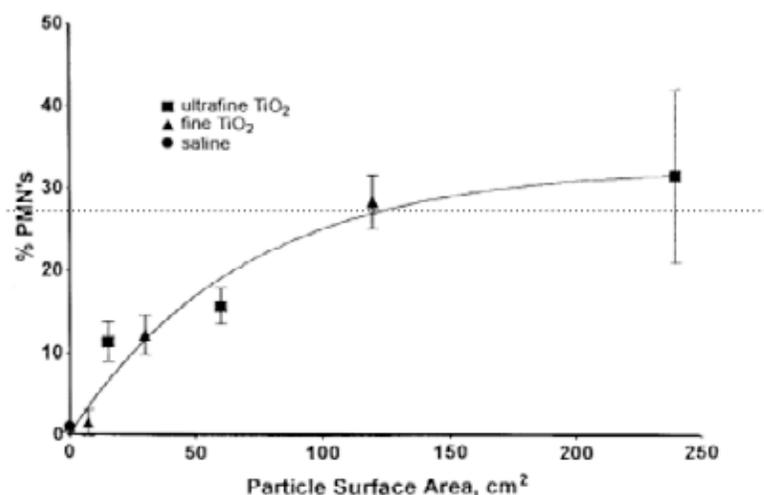
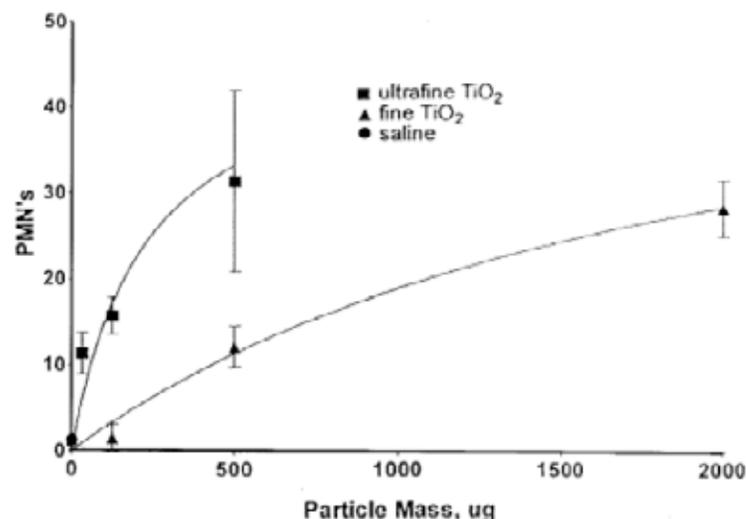


Especially for materials that are of low solubility and low elemental toxicity, e.g. Ti, Zr, Ba, Au, polymers, fullerenes

- Toxicity of ultrafine TiO₂ appears much higher than fine TiO₂ *per unit mass*
- Toxicity is equivalent when *surface area* is the exposure metric

Measured polymorphonuclear neutrophils in lung lavage fluid, an index of inflammation

Oberdorster, Int Arch Occup Environ Health. 2001 Jan;74(1):1-8.



Catalysis of Oxidative Stress

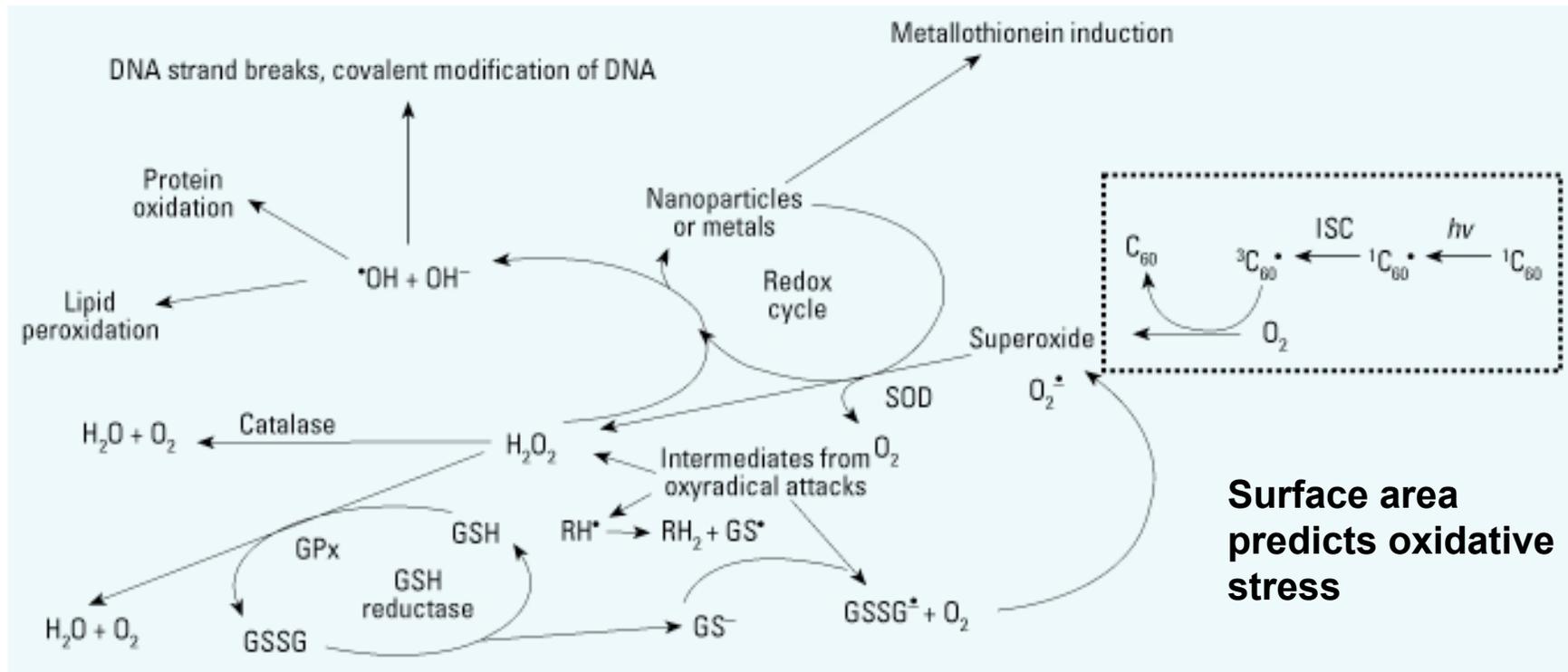


Figure 6. NPs have been shown to release oxyradicals [pictured here is the mechanism of C_{60} as determined by Yamakoshi et al. (2003)], which can interact with the antioxidant defense system. Abbreviations: GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; ISC, intersystem crossing; R, any organic molecule; SOD, superoxide dismutase. In addition to fullerenes, metals such as cadmium, iron, or nickel quantum dots, or iron from SWNT manufacturing, could also act in Fenton-type reactions. Phase II biotransformation, ascorbic acid, vitamin E, beta carotene, and other interactions are not shown.

From Oberdorster 2006

Other Mechanisms Leading to Oxidative Stress



- Several other mechanisms that lead to oxidative stress
- Same end point--oxidized biomolecules

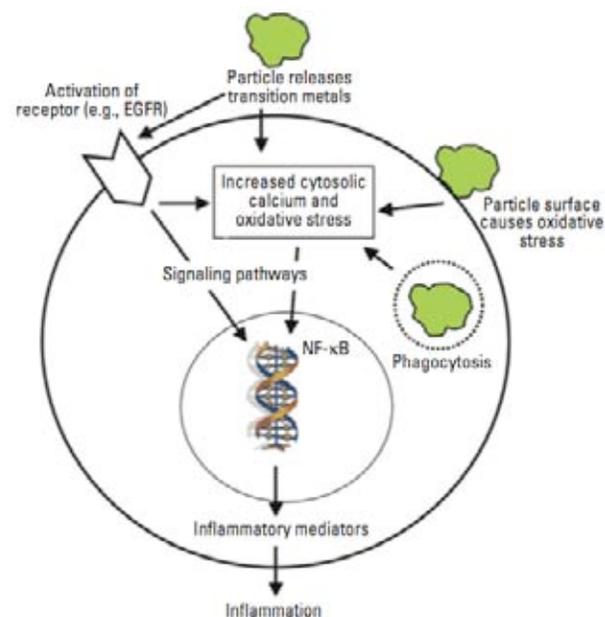
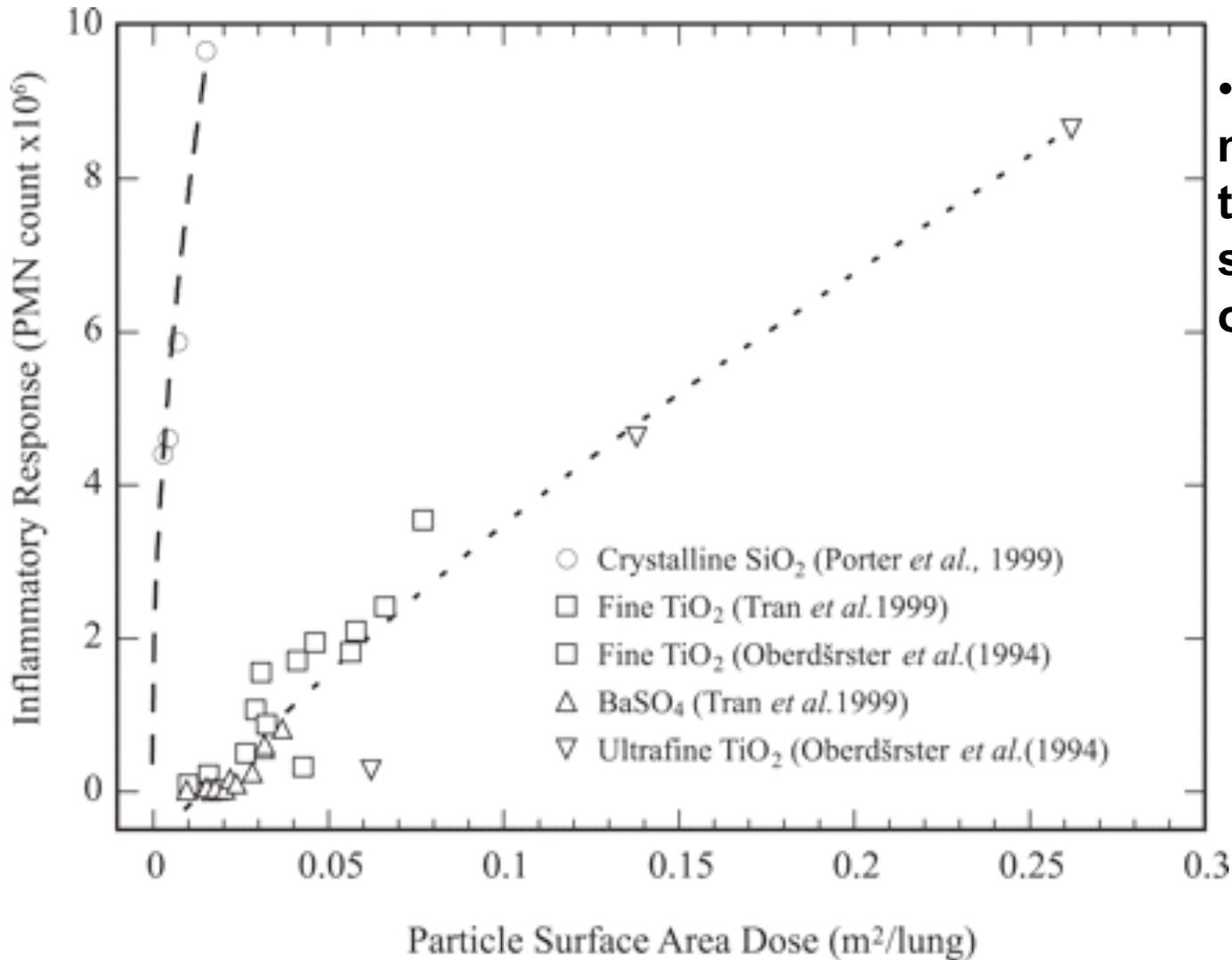


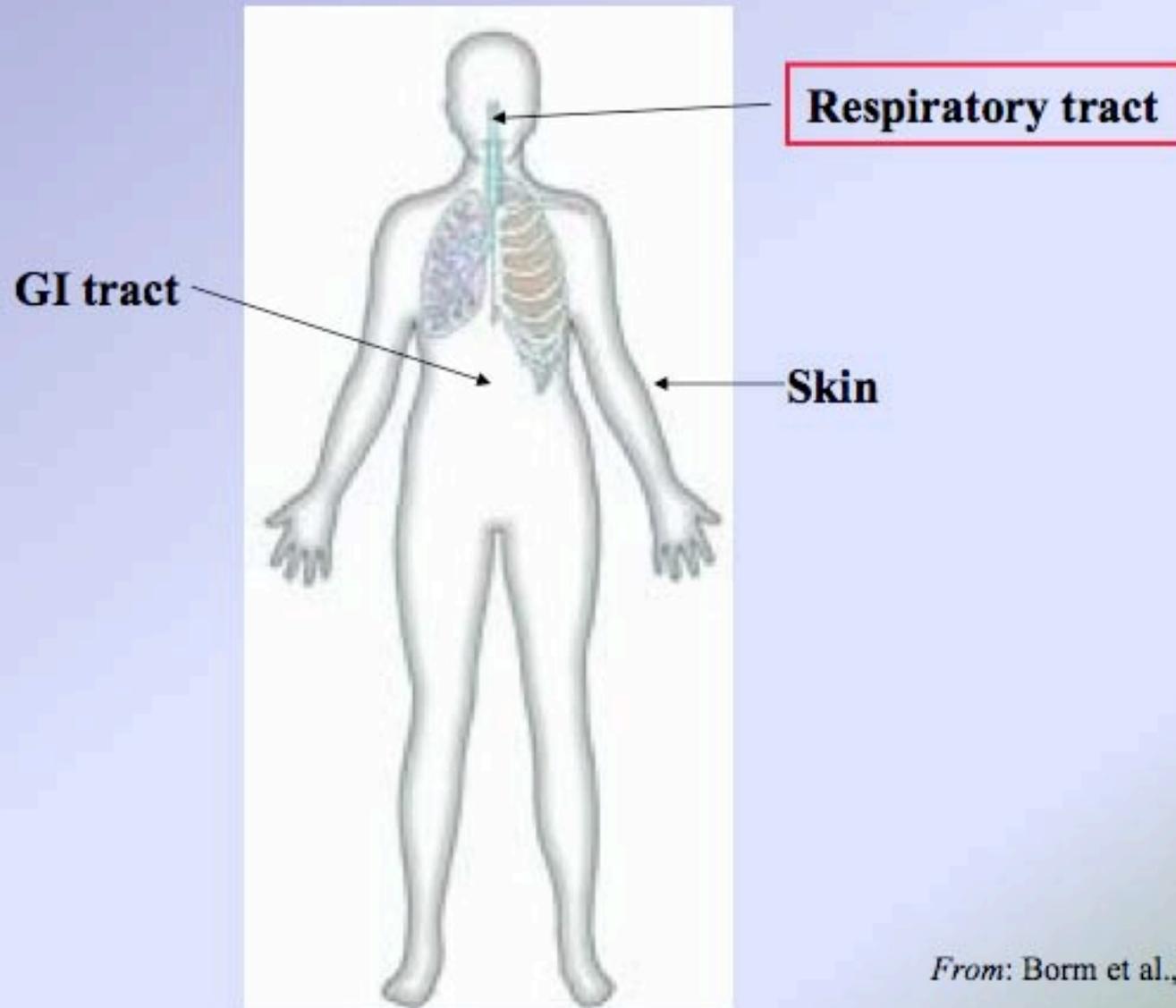
Figure 3. Hypothetical cellular interaction of NSPs (adapted from Donaldson and Tran 2002). EGFR, epidermal growth factor receptor. Inflammation and oxidative stress can be mediated by several primary pathways: *a*) the particle surface causes oxidative stress resulting in increased intracellular calcium and gene activation; *b*) transition metals released from particles result in oxidative stress, increased intracellular calcium, and gene activation; *c*) cell surface receptors are activated by transition metals released from particles, resulting in subsequent gene activation; or *d*) intracellular distribution of NSPs to mitochondria generates oxidative stress.

Dose of Perspective



• Nanoquartz is much more toxic than other low solubility nano-oxides

Exposures to Nanomaterials: Most Likely Routes

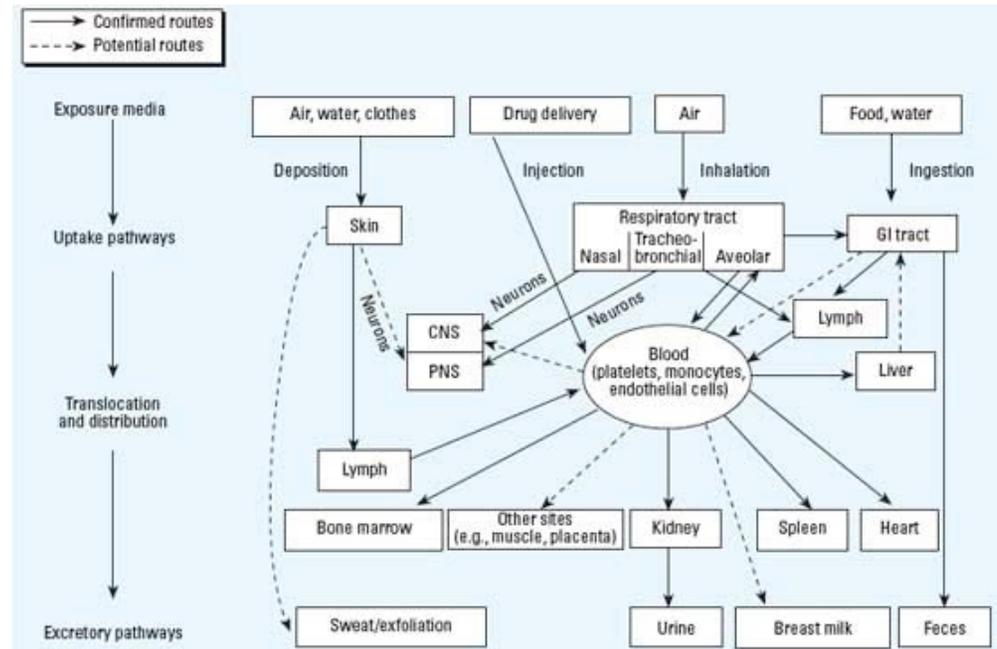


From: Borm et al., 2006

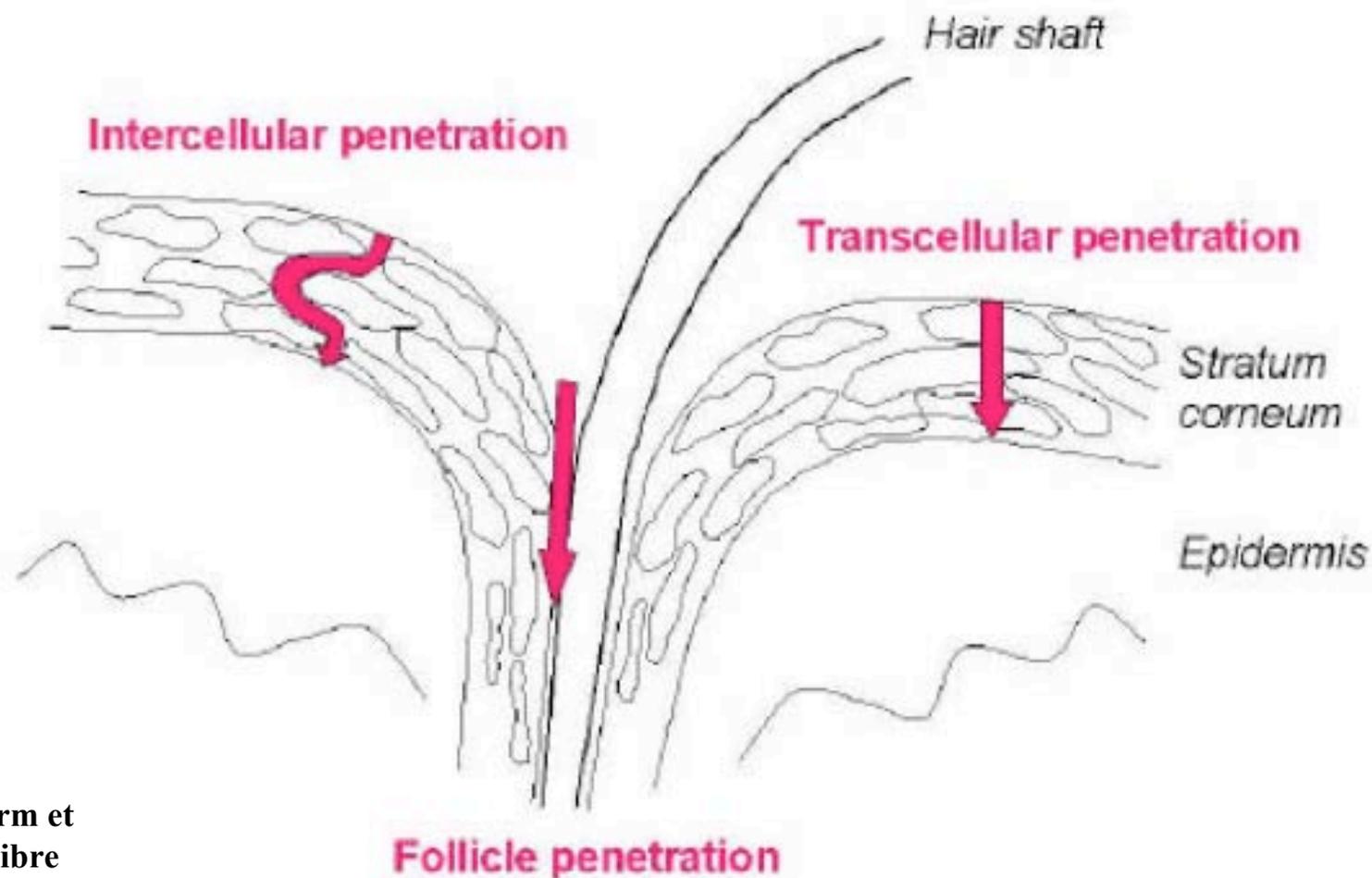
Distribution Across Anatomical Barriers



- Nanoparticles may bypass normal body barriers to distribution!
 - Through intact skin—sometimes
 - Through the GI epithelium—yes!
 - Through the respiratory tract epithelium—Yes
 - Up along nerve axons from the nose to brain—Yes
 - Across the placenta! Barrier—Possibly
 - Through the blood-brain barrier—Maybe
 - Through the blood-testes barrier—Probably



Can Nanoparticles penetrate the skin?



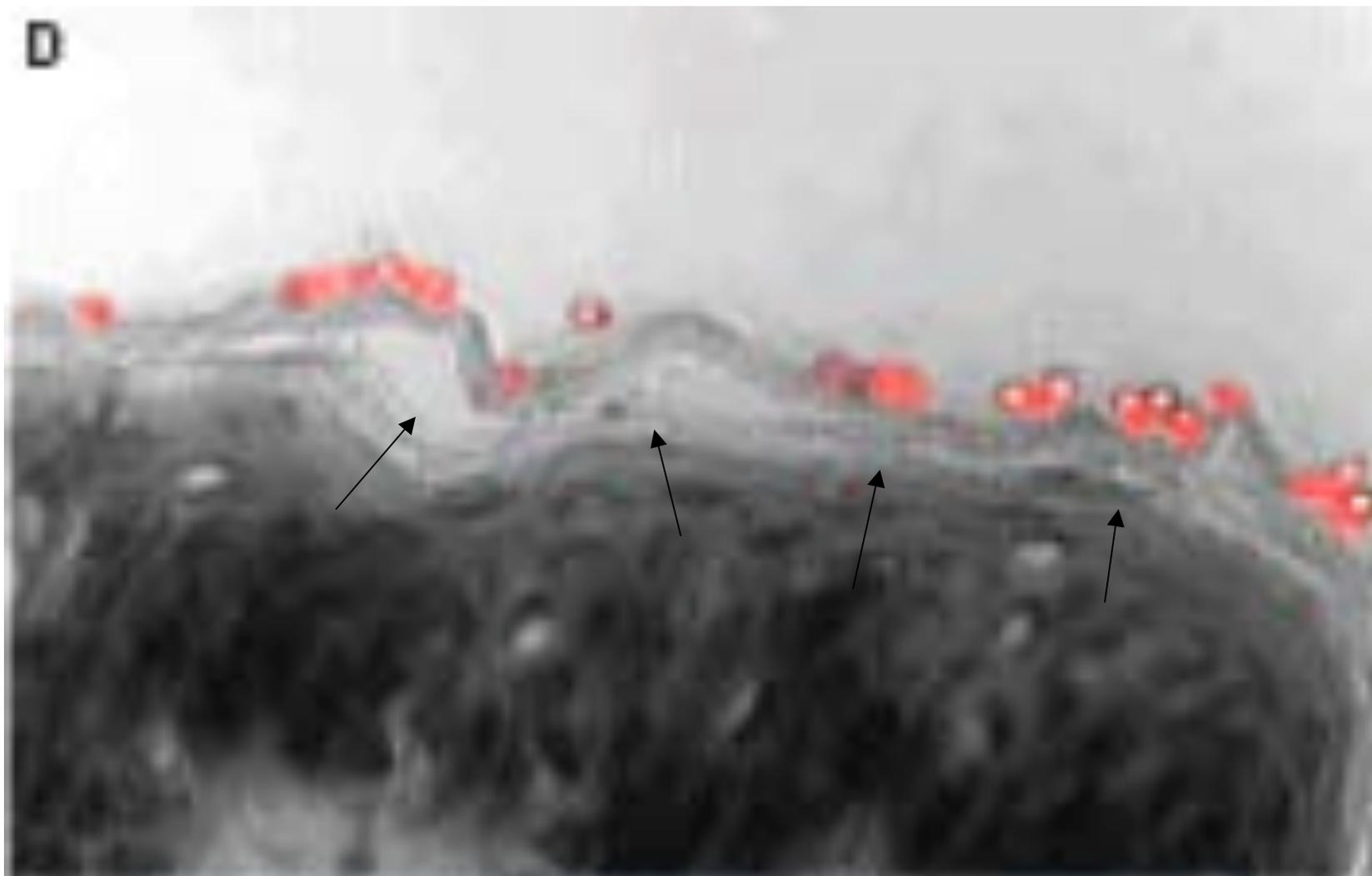
•From Paul Borm et al, J Part and Fibre Tox, 2007

Can Nanoparticles Penetrate the Skin?

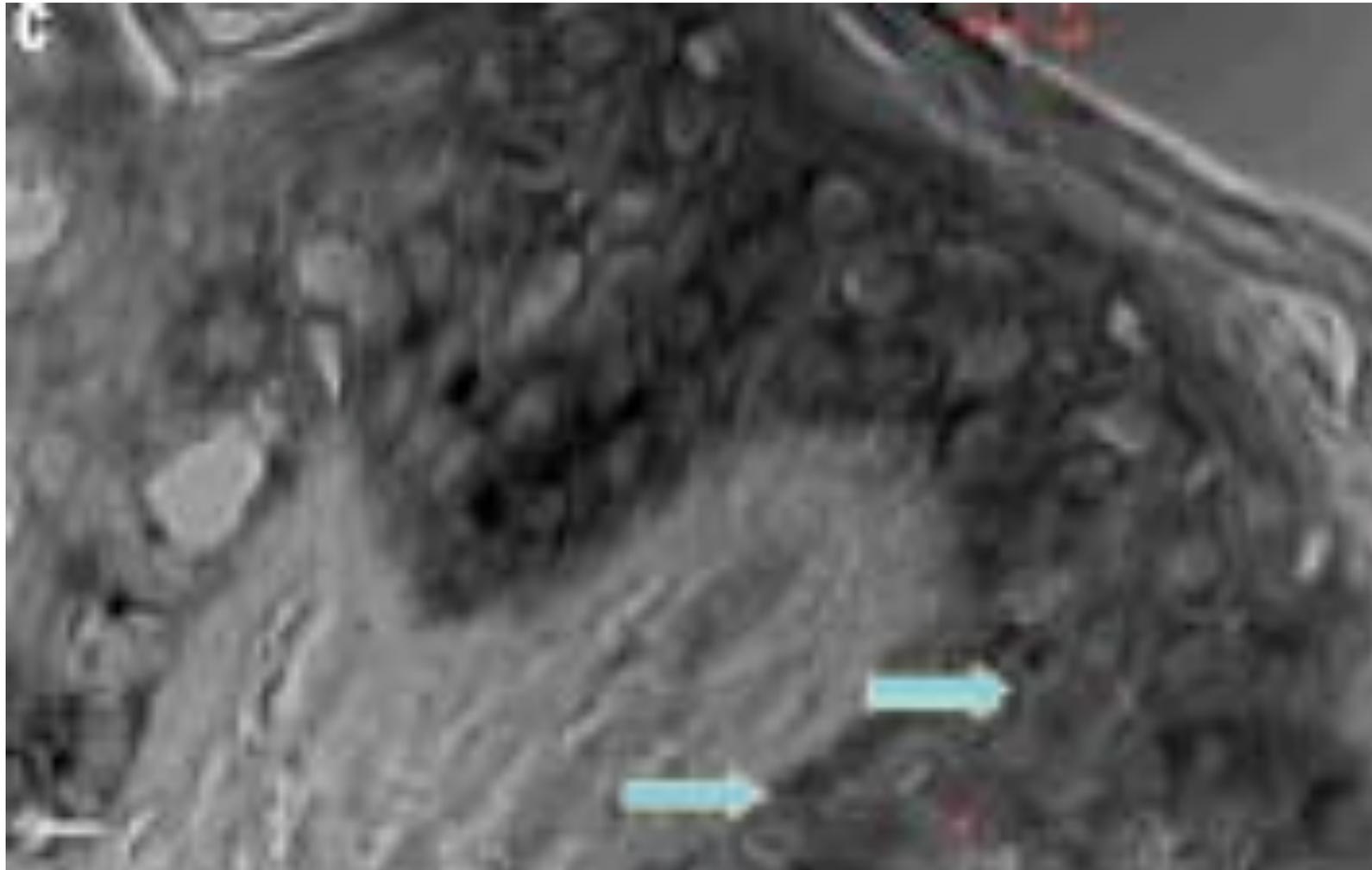


- **Static application models never show penetration**
- **Flexed skin models show some penetration**
 - After flexion/ extension of skin, smaller fluorescent dextran beads 500, 1000, 4000 nm penetrate epidermis reaching dermis**
 - (Tinkle, et al. Environ Health Perspect. 2003; 111:1202-1208)

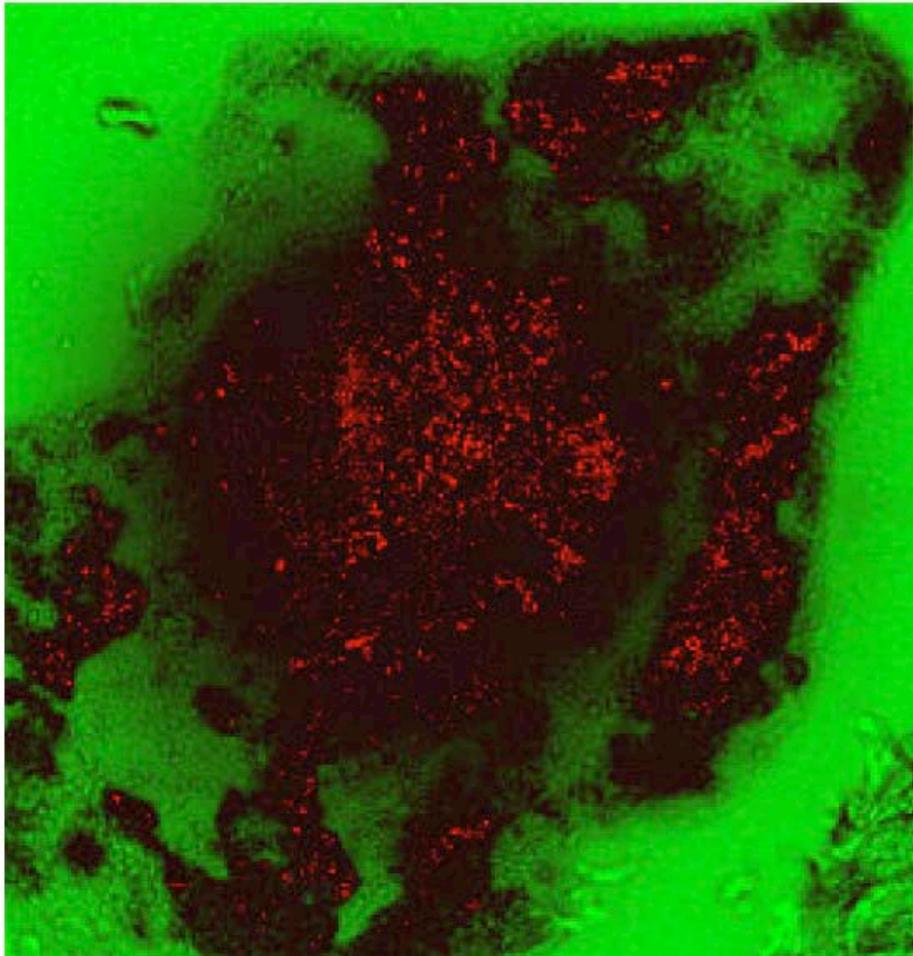
4000 nm Dextran beads remain on skin surface after flexion/extension



500nm beads in epidermis after 30 minutes of flexion/extension



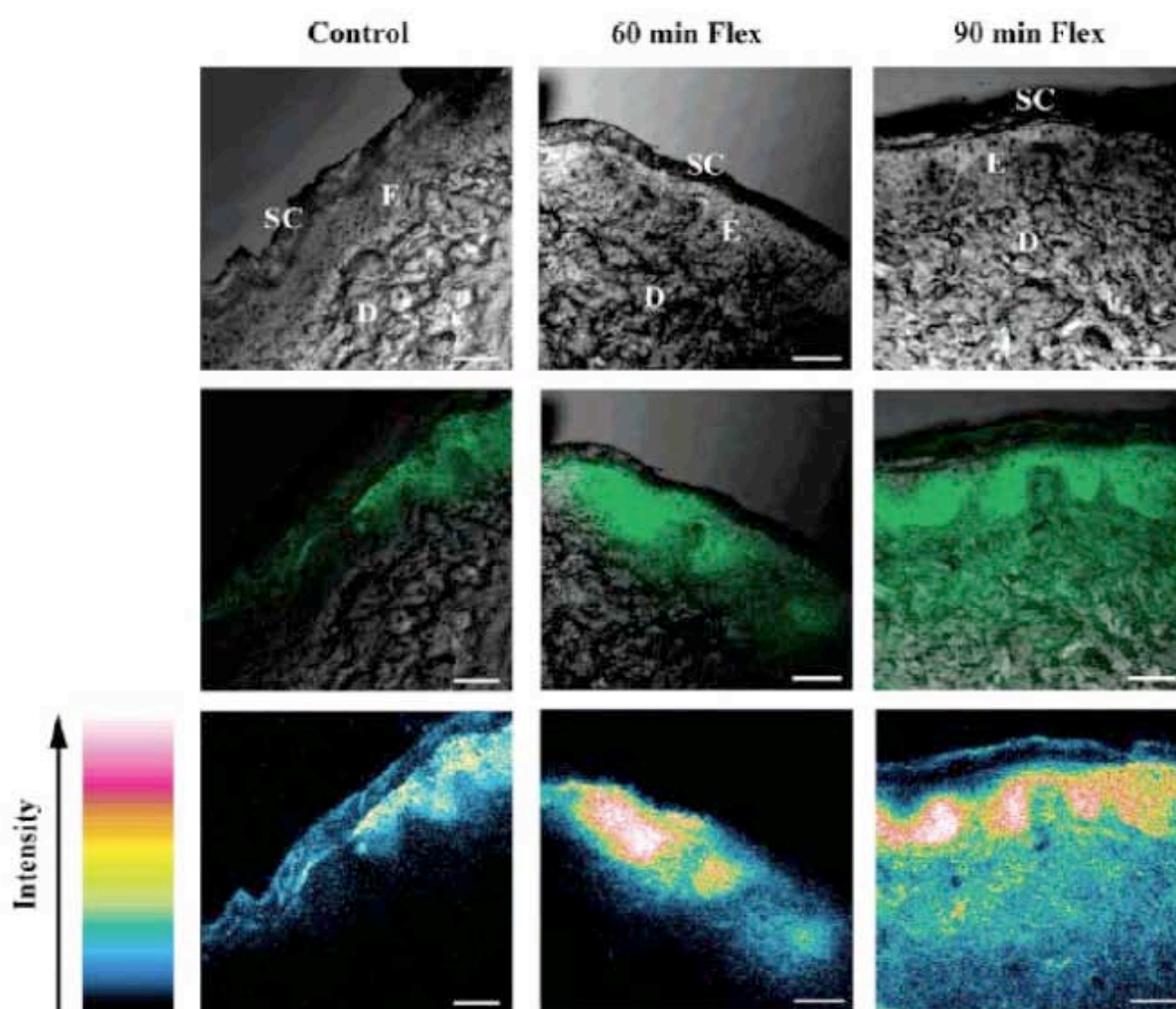
TiO₂ certainly gets into hair follicles



100

- **Red fluorescence indicates TiO₂ nanoparticles (sunscreen) trapped in hair and oil pores in skin.**
- **Used “tape stripping” on live humans**
- **Stratum corneum:** presence of titanium dioxide NP
- **Follicles:** in approximately 10% of follicles fluorescence was observed
- **Interfollicular epidermal tissue below the stratum corneum:** absence of titanium dioxide NP

Fullerene Skin Penetration with Flexion



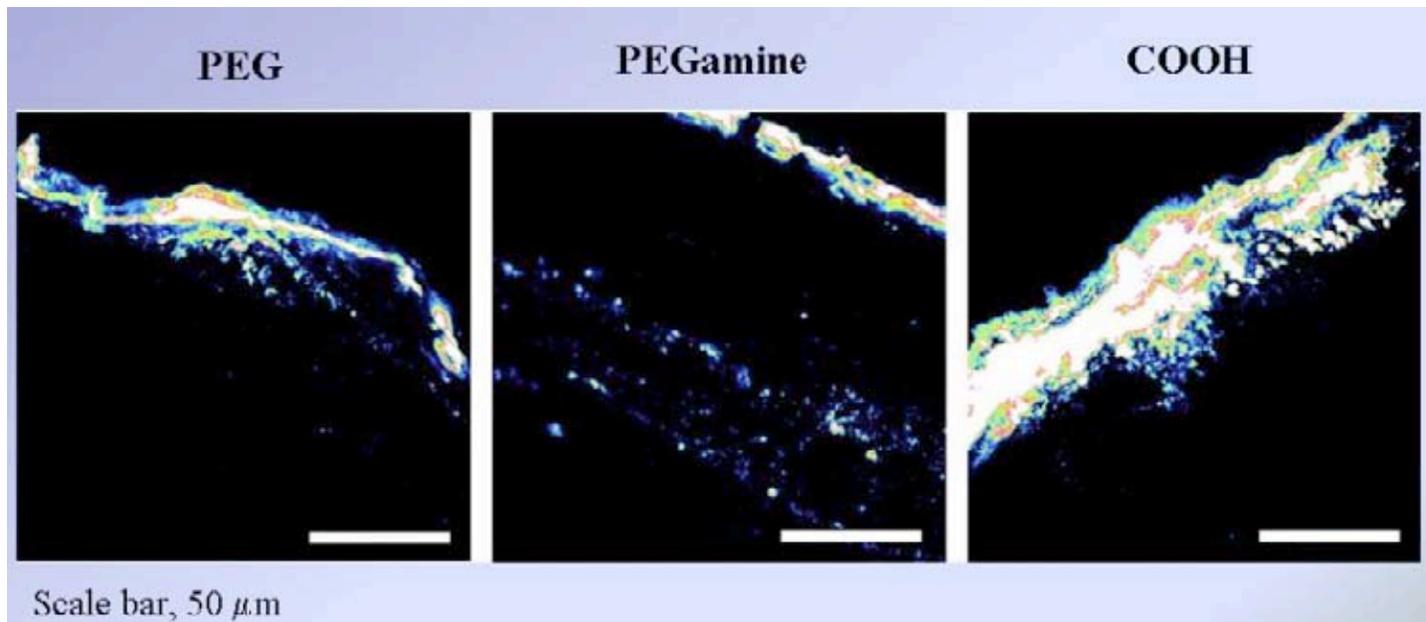
**Peptide-coated
fullerenes do
penetrate the skin
following flexion
and extension.**

**Jillian G. Rouse,^{†,‡}
Effects of Mechanical
Flexion on the
Penetration of
Fullerene Amino
Acid-Derivatized
Peptide Nanoparticles
through Skin
NANO LETTERS
2007, Vol. 7, No. 1
155-160**

Quantum Dots

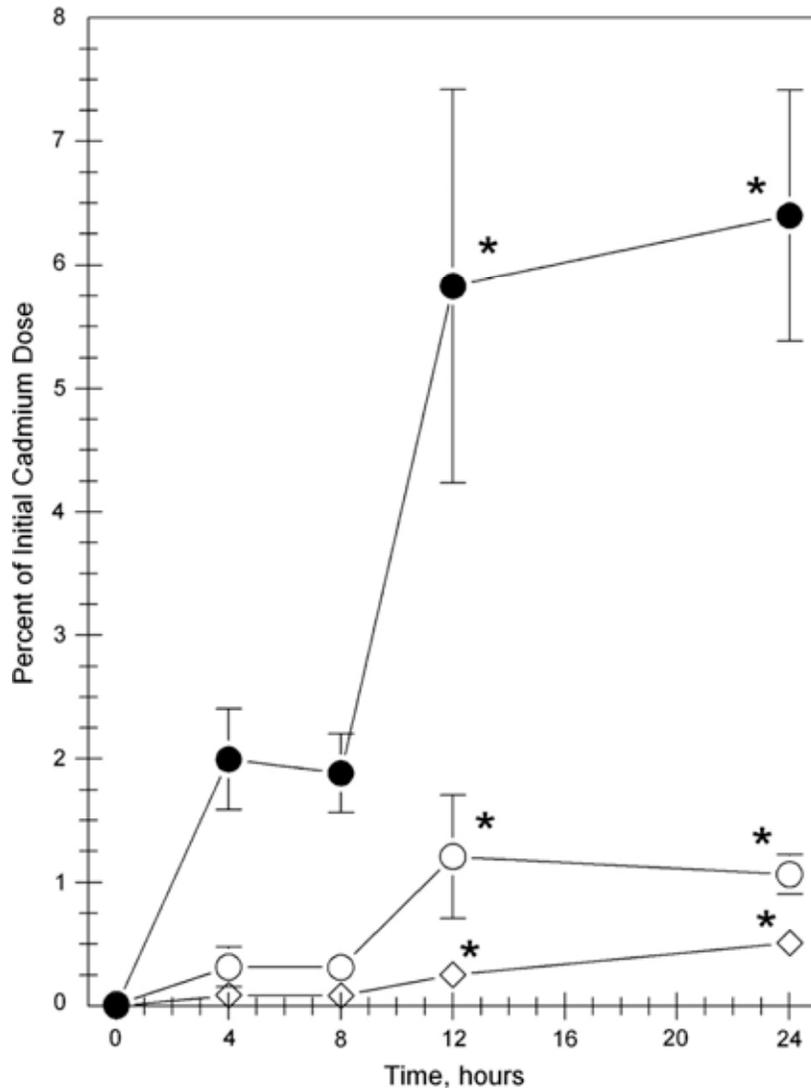


- **CdSe quantum dots can penetrate damaged skin to a limited extent (Ryman-Rasmussen 2006, Monteiro-Riviere 2008)**



- **Penetration is limited, and varies strongly based on the surface charge of the QDs**

QDs injected into the skin do circulate

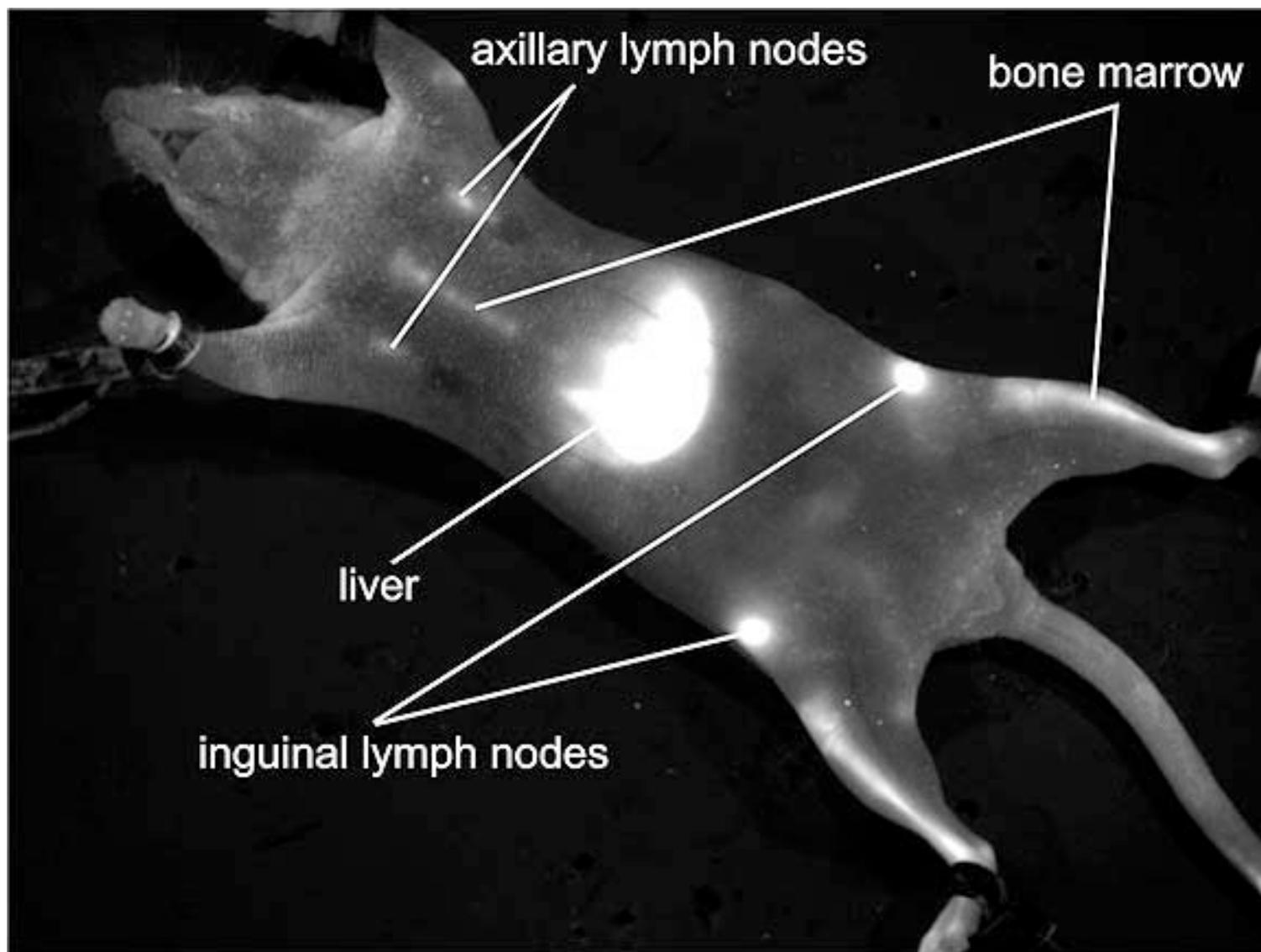


After 24 hours

- Liver
- Lymph
- Kidney

•Migration of Intradermally Injected Quantum Dots to Sentinel Organs in Mice, Gopee et al, 2007

Bioaccumulation– Quantum Dot Mouse



•B. Ballou, BC
Lagerholm, LA
Ernst, M
P.Bruchez,AS
Waggoner;
Bioconjugate
Chem. 2004, 15,
79-86



Translocation From the Lung

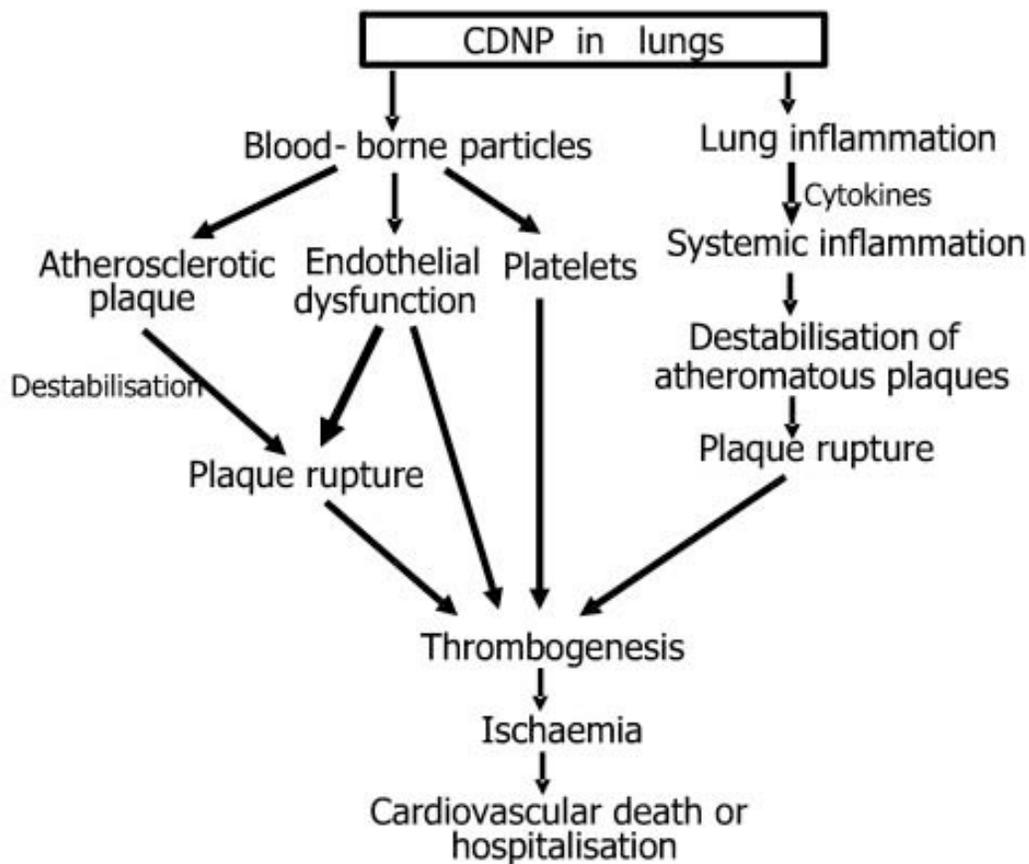
To blood, lymph and beyond!

Why do we care about translocation?



- **Bad Karma!**
 - Translocation of asbestos fibers into the mesothelium leads to mesothelioma
 - Translocation of CDNP may be basis for some of the systemic toxicity these materials induce, including cardiovascular toxicity

Hypothetical Mechanisms for Cardiovascular Toxicity of CDNP



May or may not require that particles translocate from lung

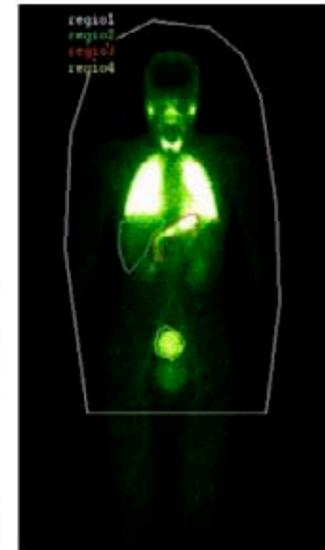
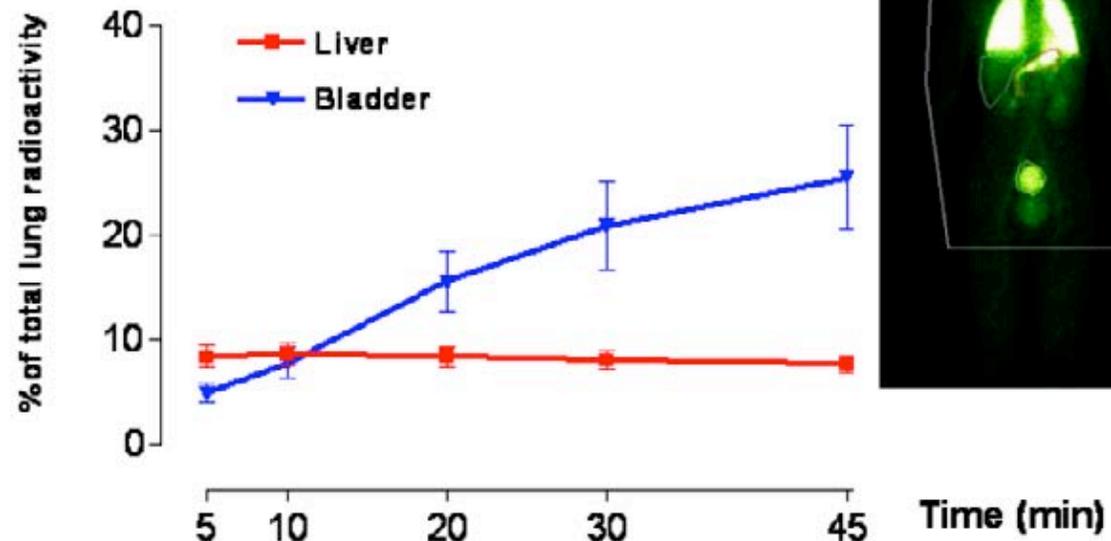
Donaldson et al 2005

Translocation to lung to blood



- Very controversial due to flawed early studies
- One early study showing very rapid translocation was flawed due to rapid dissolution of the particles, **overestimating translocation**

• Example 1:
involving 25 nm
 Tc^{99} , carbon coated
particle



Likely slower and size dependent



- Kreyling studied the transport of radioactive irridium particles through lung
- 80 nm--0.1% translocated through the lung and ended up in the liver
- 15 nm--0.5% translocated, a 5x increase

Increased by lung inflammation



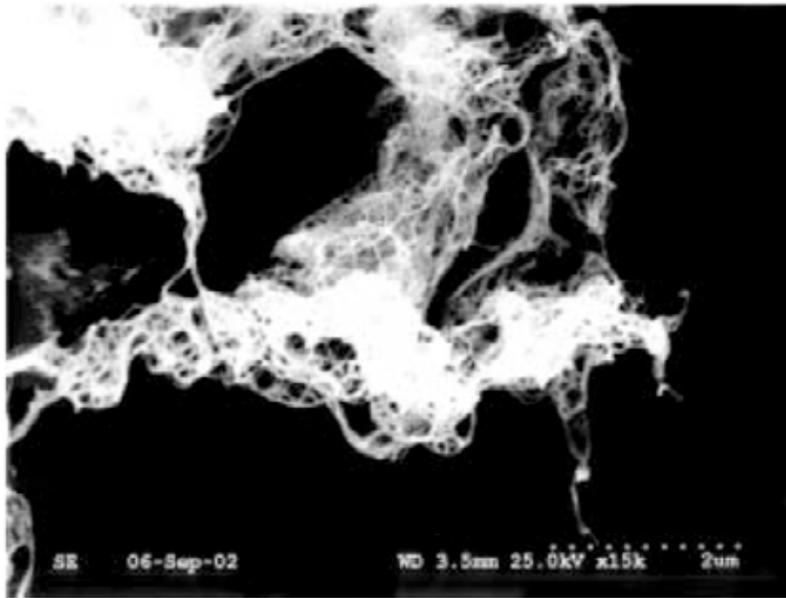
- “ Only a small fraction of intratracheally instilled UFPs can pass rapidly into systemic circulation”
- This translocation is markedly increased following LPS pretreatment.
- **Pulmonary inflammation seems to play a major role in enhancing the extrapulmonary translocation of particles.**
- Relevant to epidemiology outcome which suggests that the elderly and people with pre-existing cardiorespiratory disease are at a higher risk of particles-induced injuries.”

•Chen et al 2006

Newest NIOSH Research



- **Carbon nanotubes do penetrate the lungs of mice and migrate to the pleura, just like asbestos**

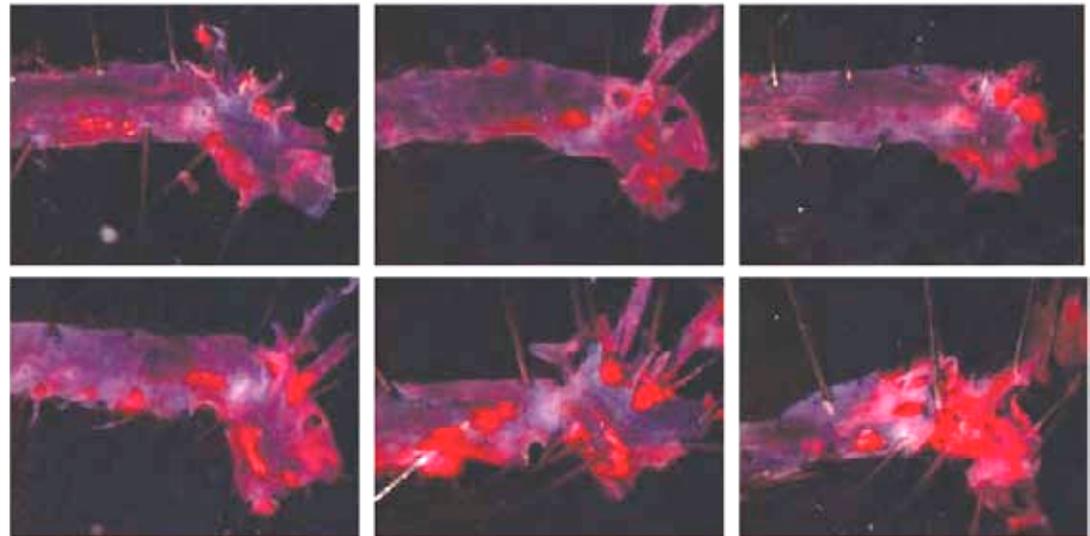


Cardiovascular Toxicity of CNTs Instilled In The Respiratory Tract



- Li Zheng of NIOSH, March 2007
 - Intraparyngeal dosing of SWCNT in mice resulted in oxidative stress in aorta and heart tissue and damaged mtDNA in aorta
 - Accelerated atherosclerosis

Control aortas



SWCNT exposed aortas

Lung Dosed Nano TiO₂ Interferes with systemic arterial function

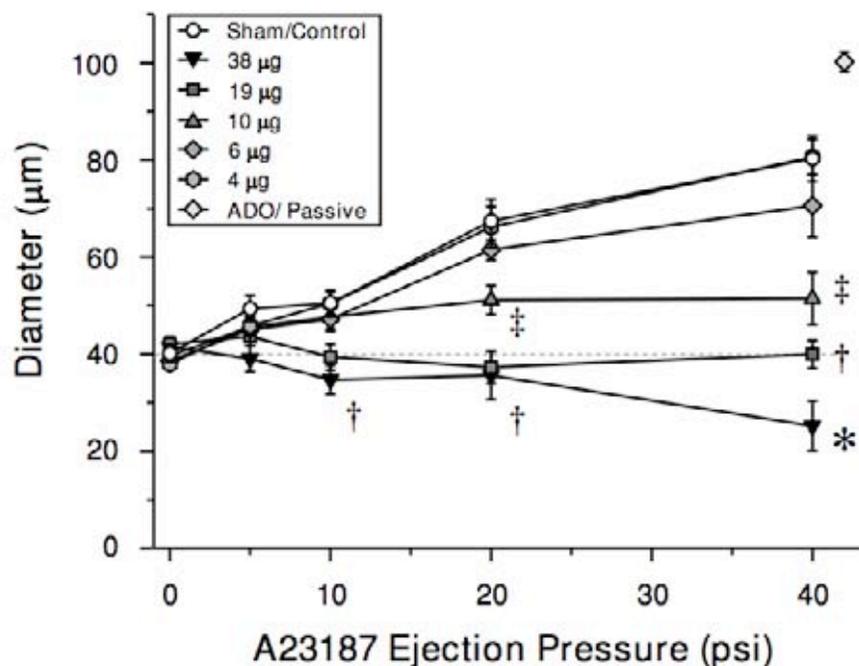


Figure 6
Ultrafine TiO₂ inhalation impairs systemic arteriolar dilation 24 hours after exposure in a dose-dependent manner. Sham/Control, n = 8; 38 µg, n = 9; 19 µg, n = 11; 10 µg, n = 8; 6 µg, n = 7; 4 µg, n = 9. Values are means ± SE. *, P < 0.05 vs. 19 µg group. †, P < 0.05 vs. 10 µg group. ‡, P < 0.05 vs. 6 µg group. Adenosine (ADO).

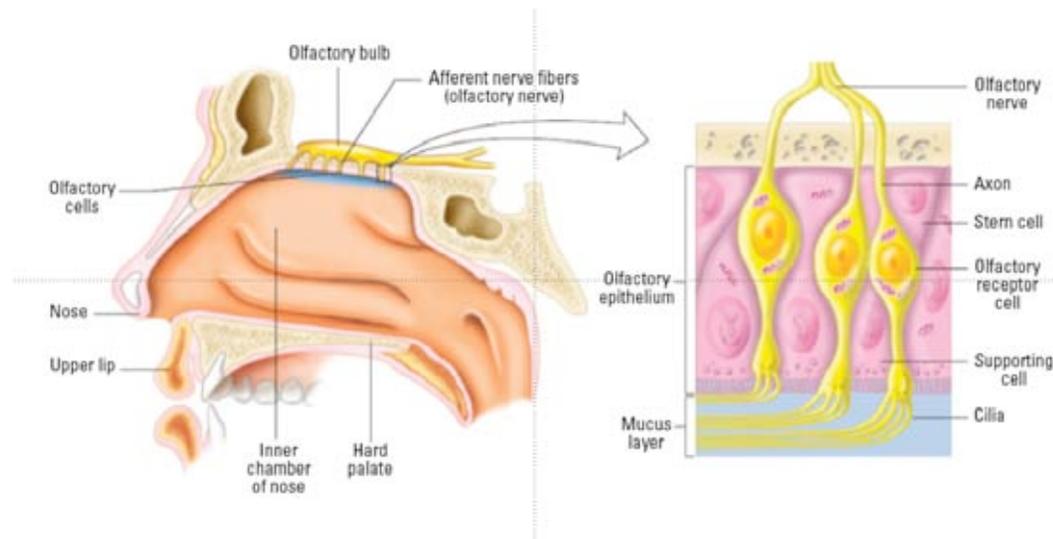
- Nano TiO₂, much more than micro TiO₂, causes systemic microvascular dysfunction (for equal mass, not true when expressed as surface area)
- Dose related
- Suppresses endothelium-dependent dilation of systemic arterioles in response to chemical dilator
- Dose that caused only very minor lung inflammation
- Due to rapid accumulation of PMN leukocytes along microvascular wall

Timothy Nurkiewicz, 2008

Translocation from Nose to Brain



- **Known that polio virus particles can enter the brain via the olfactory nerves since 1941**
- **Studies in monkeys with intranasally instilled gold ultrafine particles (UFPs; < 100 nm) and in rats with inhaled carbon UFPs (36 nm) suggested that solid UFPs deposited in the nose travel along the olfactory nerve to the olfactory bulb**



MnO Translocation from Nose to Brain



- Inhalation of nano MnO resulted in increase in brain Mn levels, particularly in the olfactory bulb
- Might be due to brain uptake from the blood of ingested or dissolved Mn, but hard to explain the specific targeting of the olfactory bulb
- Suggests direct olfactory transport to olfactory bulb

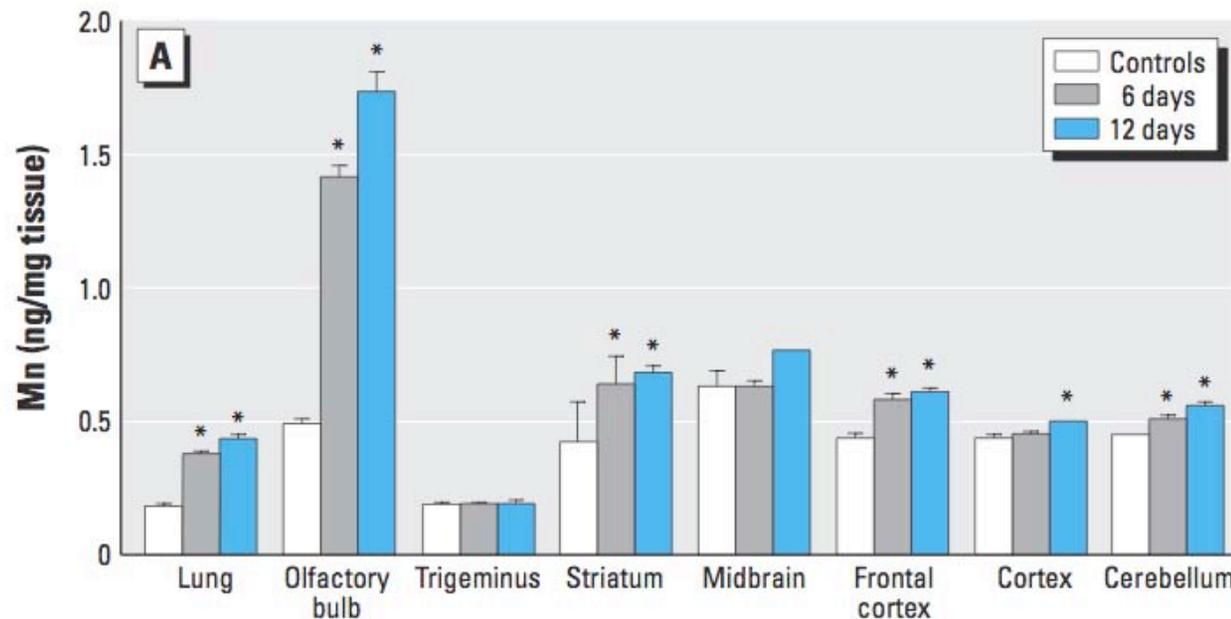
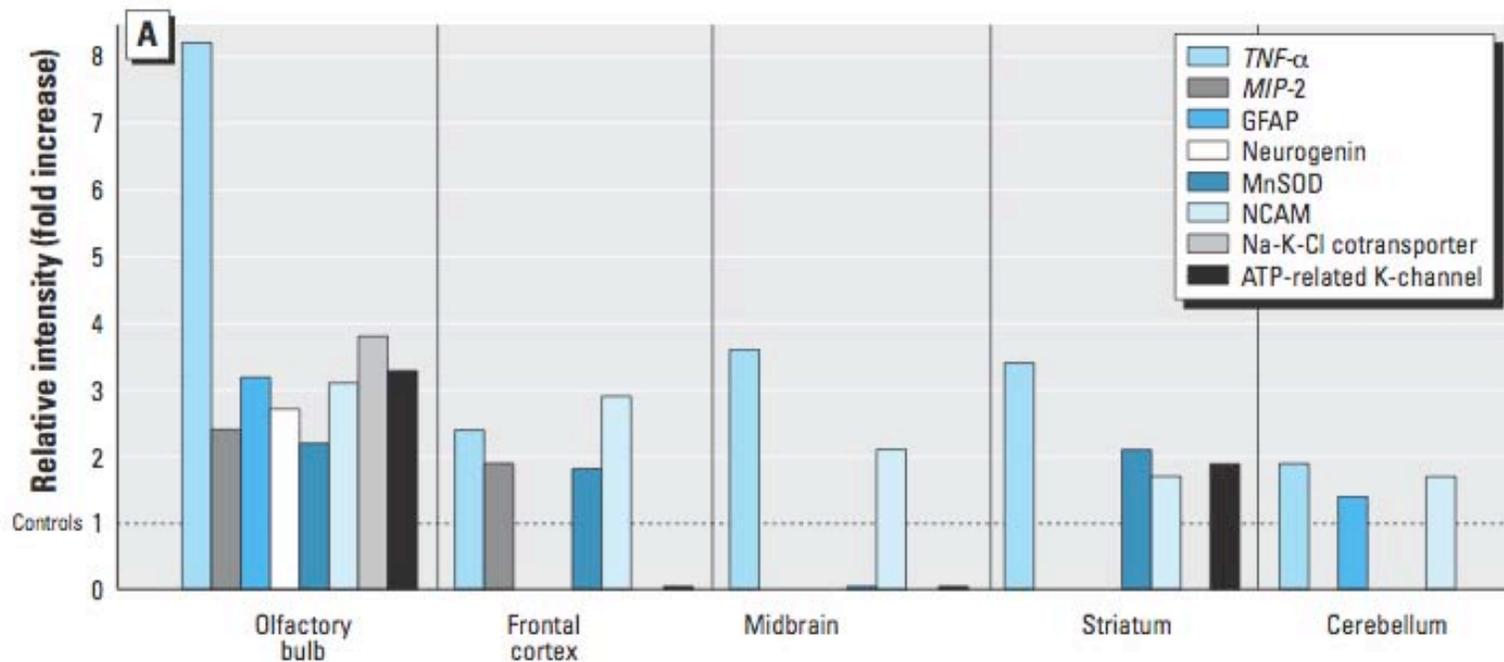


Figure 1. Mn and Fe contents in lung and brain tissues after 6 and 12 days of inhalation

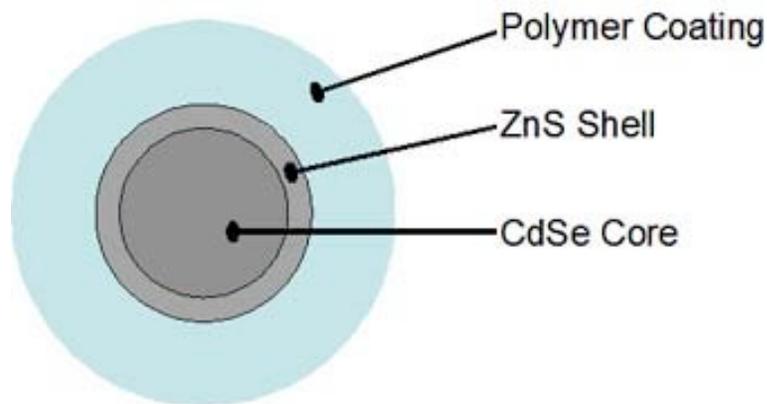
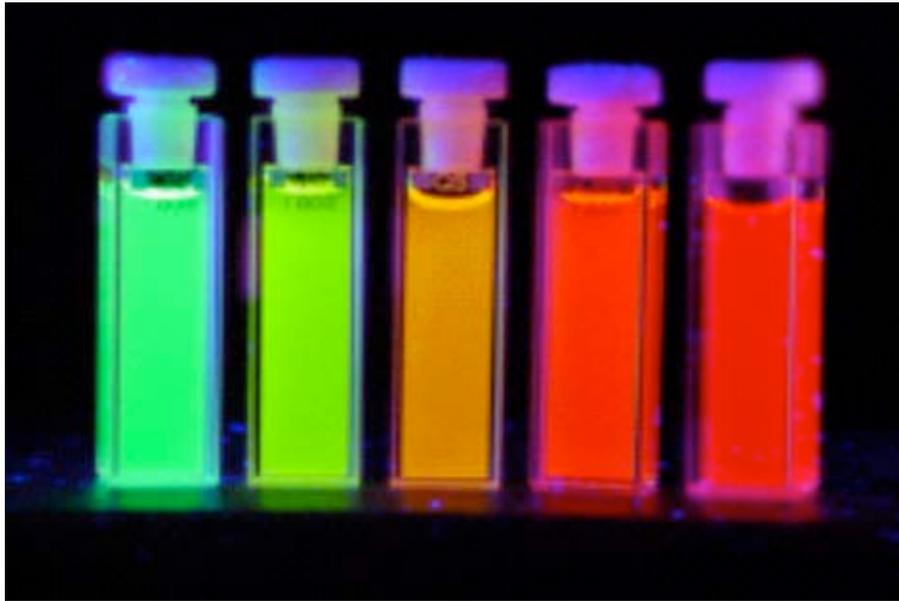
MnO in the brain



- Lead to indicators of inflammation in the brain where the MnO was deposited



Quantum Dots



- Semiconductors a few nm in size
- CdSe, CdTe, GaAs, other metals
- Fluorescence is a function of size
- Potentially very useful in medicine and science
- All start with metal core, some have metal shells then usually plastic coating, sometimes ligands attached to core

Possible Mechanisms of Toxicity



- 1. Redox reaction to release cytotoxic Cd²⁺ ion
--CdSe + O₂ yields Cd²⁺ & SeO₂**
- 2. Oxidative stress due to formation of singlet oxygen and superoxide ion, leading to oxidation of cell components**
- 3. Something else entirely**

Naked Quantum dots are toxic



- **“Naked” QDs induce damage to the plasma membrane, mitochondria, and nucleus, leading to cell death.**
- **When coated with proteins or biocompatible polymers, QDs are not deleterious to cells and organisms.**
- **However, when QDs are retained in cells or accumulated in the body for a long period of time, their coatings may be degraded, yielding "naked" QDs.**

Quantum dots Cho et al 2007

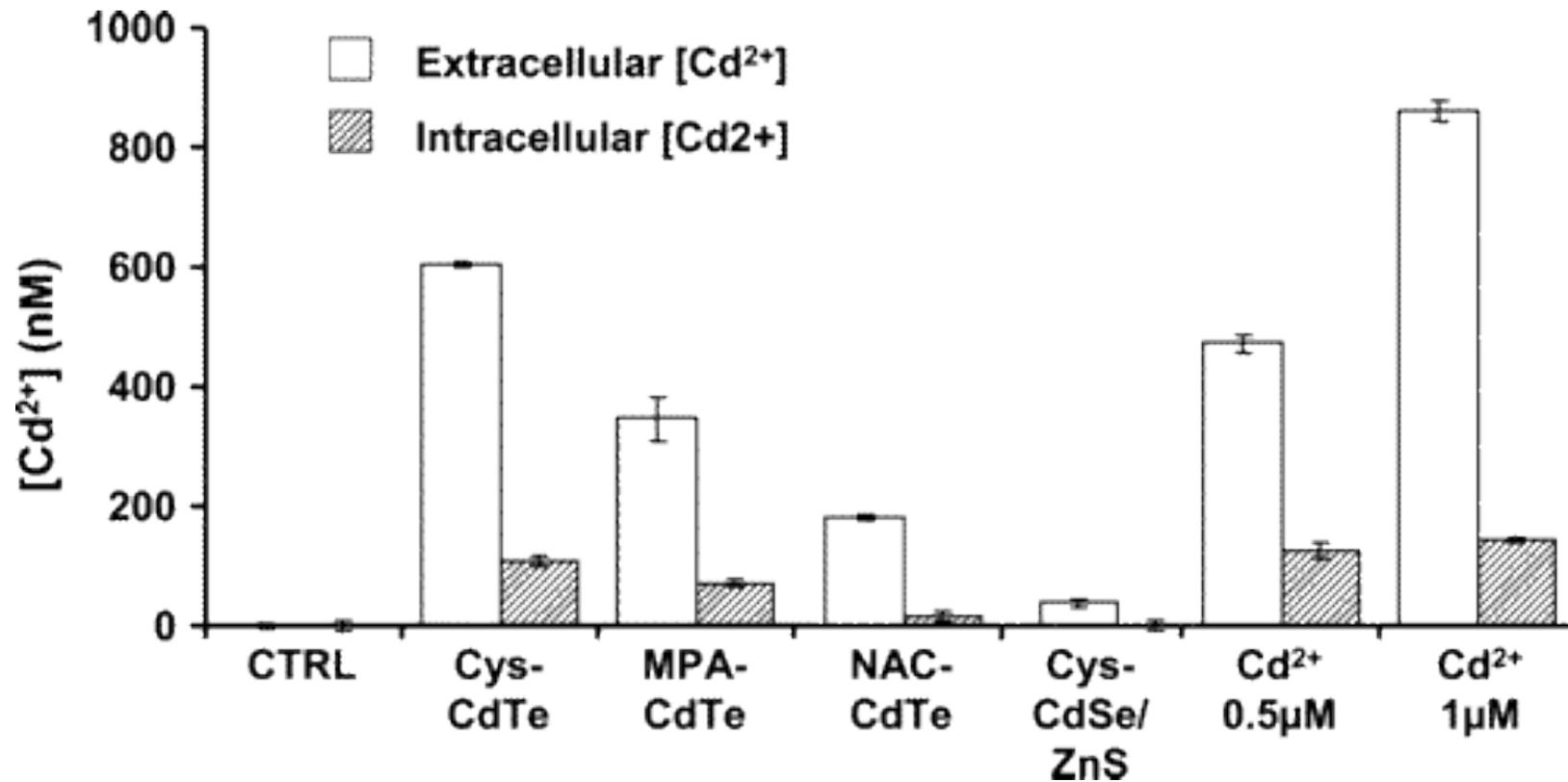


- **Several studies suggest that the cytotoxic effects of quantum dots (QDs) may be mediated by cadmium ions (Cd^{2+}) released from the QDs cores.**
- **The objective of this work was to assess the intracellular Cd^{2+} concentration in human breast cancer MCF-7 cells treated with cadmium telluride (CdTe) and core/shell cadmium selenide/zinc sulfide (CdSe/ZnS) nanoparticles capped with mercaptopropionic acid (MPA), cysteamine (Cys), or N-acetylcysteine (NAC) conjugated to cysteamine.**

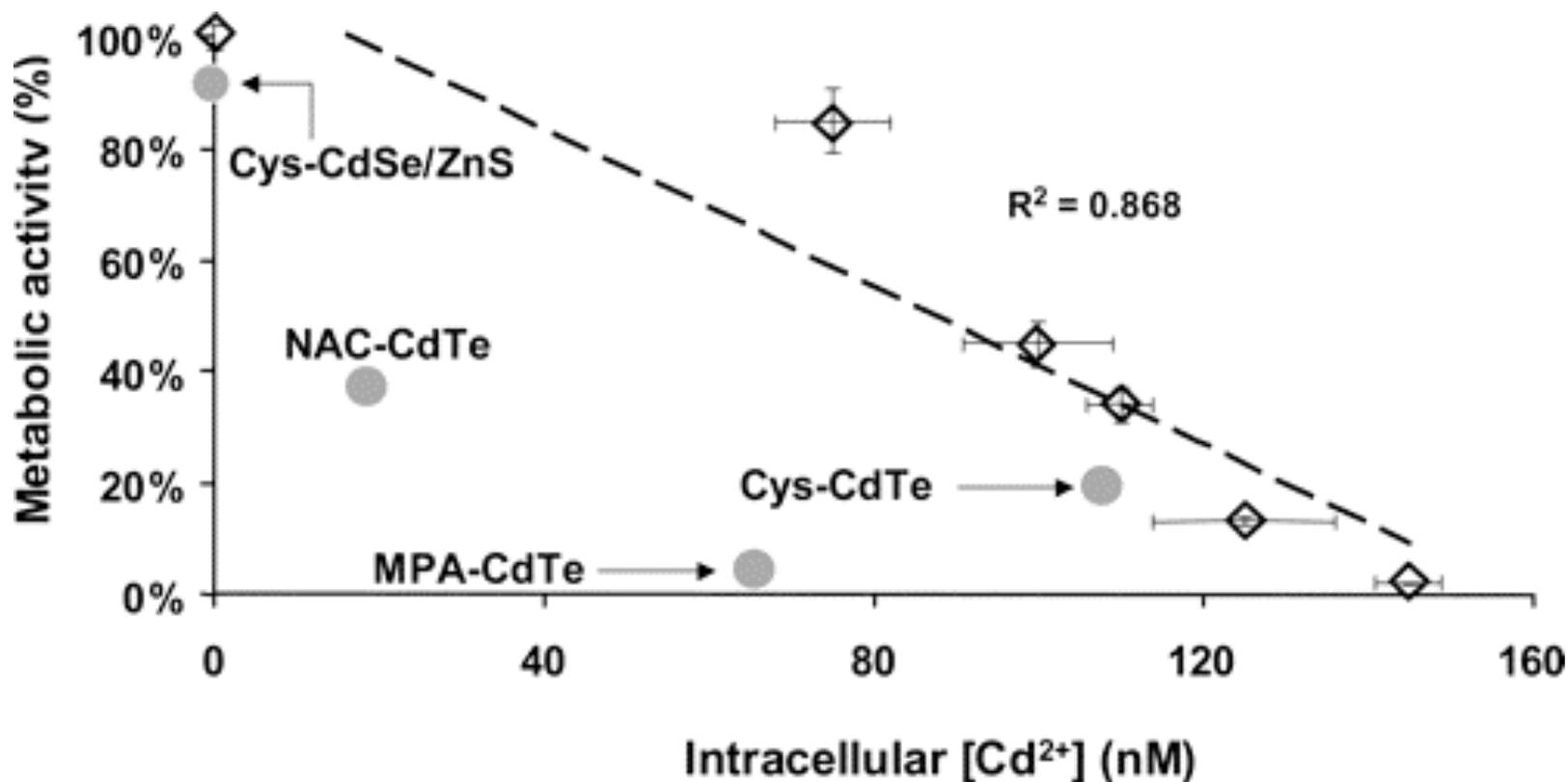
Different CdTe ND Dissolve to Differing extents



- Coatings radically alter Cd²⁺ levels

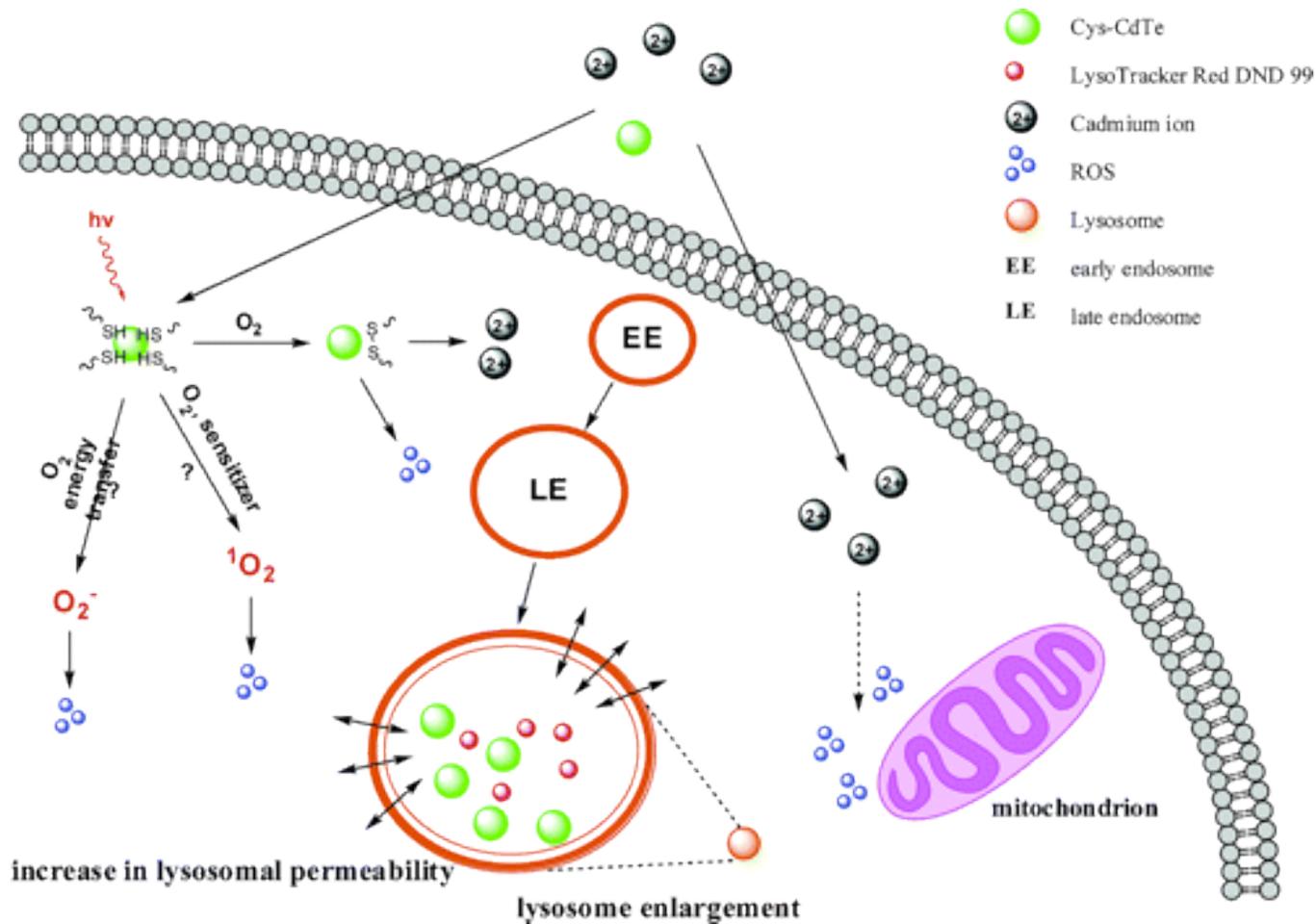


Cd²⁺ does not account for total toxicity



- Partially driven by Cd ion and partially by catalysis of oxidative stress associated with intact particle

Dual Mechanisms: CdTe Toxicity

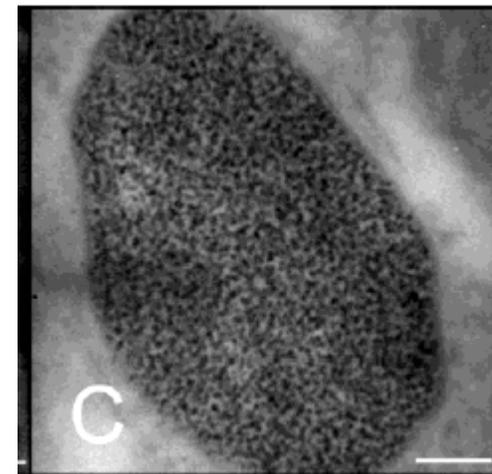
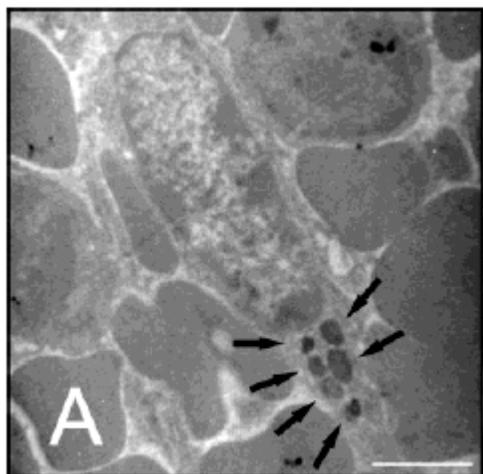
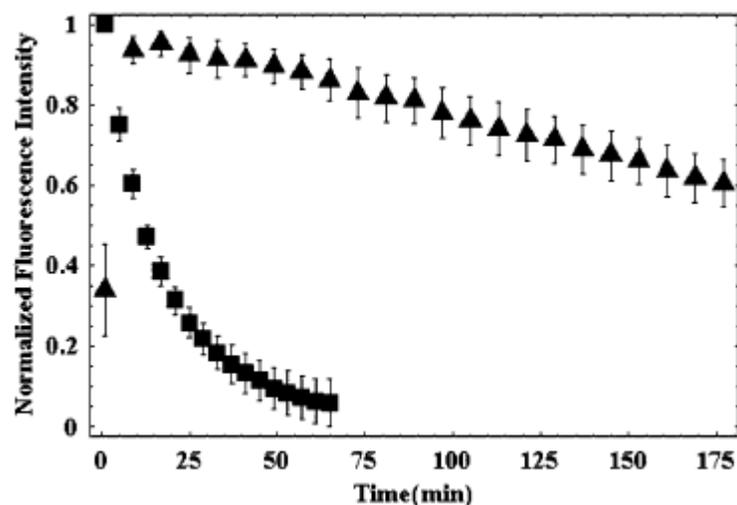


- ROS derived from Cd ion and intact particles

Elimination



- MPEG-coated Quantum Dots injected IV
- Two coatings, 750 and 5000 MW, differing half-lives
- Deposition in spleen macrophage endosomes.



Noninvasive Imaging of Quantum Dots in Mice B Ballou, BC Lagerholm,
LA Ernst, MP Bruchez, and AS Waggoner
Bioconjugate Chem.; 2004; 15(1) pp 79 - 86;

Co-Cr Artificial Joint Wear Particles



- ***The effect of nano- and micron-sized particles of cobalt–chromium alloy on human fibroblasts in vitro***
 - I. Papageorgiou, C. Brown, R. Schins, S. Singh, R. Newson, S. Davis, J. Fisher, E. Ingham and C.P. Case in “Biomaterials”, March 2007
- **Artificial joints often use a Co-Cr alloy**
- **If non-coated, to extend their life, they wear by producing nanoscale Co-Cr particles**
- **Question: What is the toxicity of these nanoscale Co-Cr alloy particles relative to microscale particles**

Cell Cytotoxicity Assay



- Nanoscale particles are notably more **cytotoxic** toxic concentrations than microparticles
- Nano particles are more **genotoxic** than micro particles, especially at higher doses

TiO₂

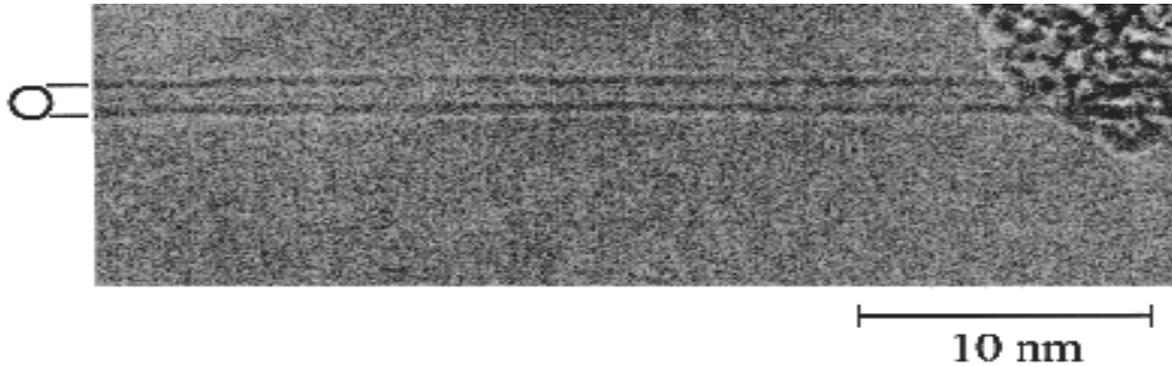


- **TiO₂--Cosmetics, clothing, sunscreen, scratch resistant sun glasses, food additive**
- **3.5 million tons per year**
- **Poorly soluble**
- **Causes surface-area related oxidative stress in rats**
- **Pulmonary overload leads to tumors in rats but not other species tested**
- **Epi evidence does not support animal models that suggest that nano TiO₂ leads to lung pathology**

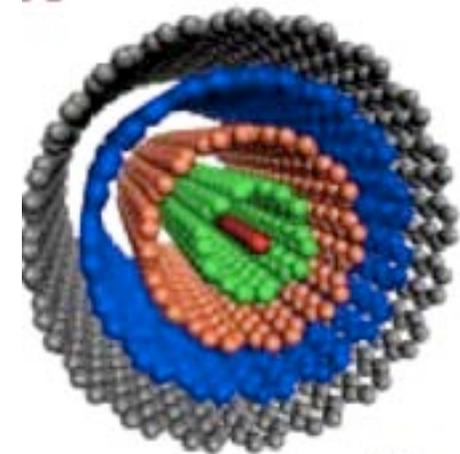
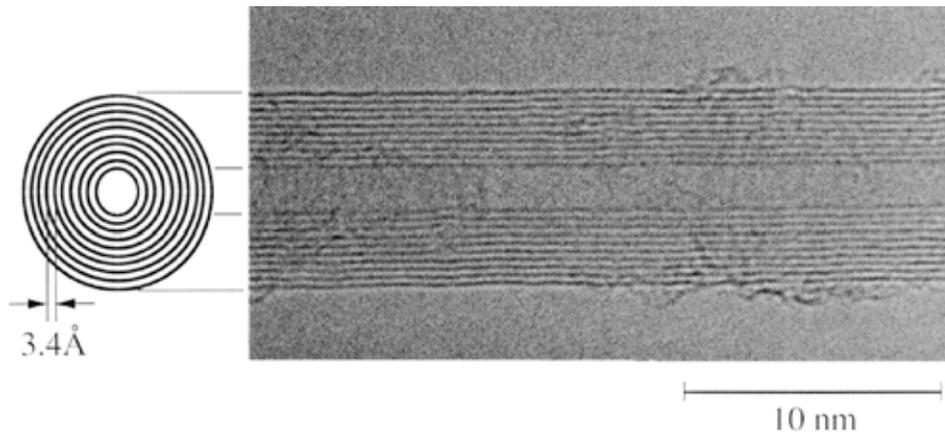
Single Tube Electron Micrographs



Single-Walled Carbon Nanotubes (SWCNTs)

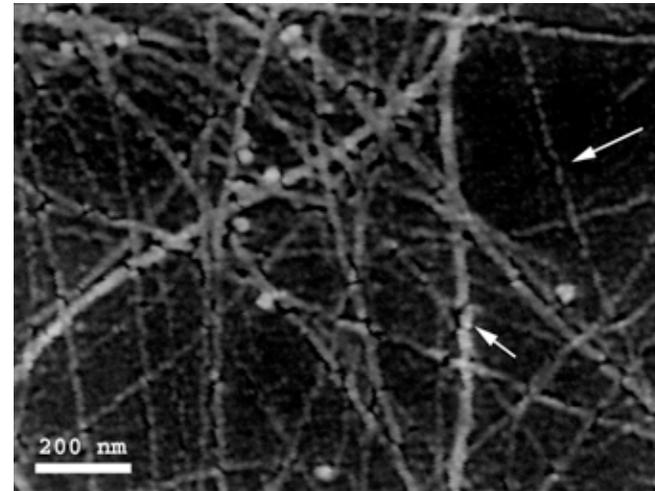
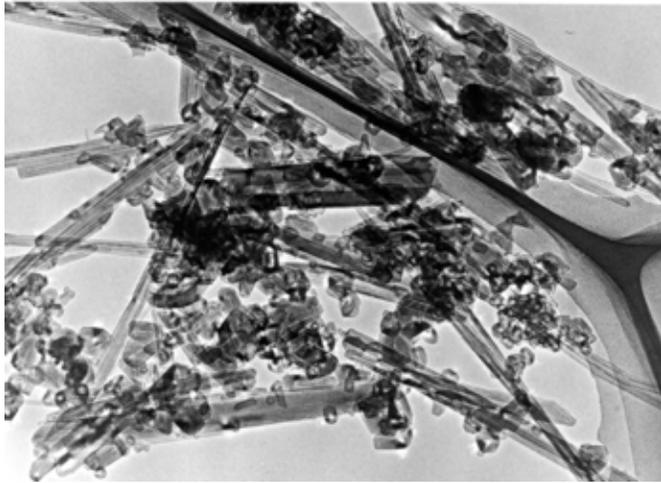


Multi-Walled Carbon Nanotubes (MWCNTs)



multi wall

What CNTs Really Look Like!



- Clumps, ropes, bundles, mats
- Residual catalyst, other carbon forms
- Very high tendency to stick together

Are CNTs Just Graphite Toxicologically?



MATERIAL SAFETY DATA SHEET

Manufacturer: Carbon Nanotechnologies, Inc. Phone: 281-492-5707
16200 Park Row Fax: 281-492-5810
Houston, TX 77084 E-mail: BusDev@cnanotech.com

Product: CNI[®] Carbon Nanotubes

Section 1 Product Identification

Chemical Name: Carbon Fullerene
Formula: Carbon
Chemical Family: Synthetic Graphite
Synonyms: Carbon Nanotubes
CAS Number: 7782-42-5 (Graphite)

Section 2 Composition and Information on Ingredients

Component	%	OSHA/PEL	ACGIH/TLV
Synthetic graphite	Up to 100%	15 mg/m ³ (total dust) 5 mg/m ³ (respirable fraction)	2 mg/m ³ TWA
Metallic impurity	Balance		

•Actual MSDS for CNTs from Carbon Nanotechnologies, Inc.

•Quoted PEL is for graphite!

What Is Special About Nanotubes?



- **May demonstrate toxicity associated with extremely high surface area typical of nanoparticles...**

AND

- **...toxicity associated with biopersistent mineral fibers...**

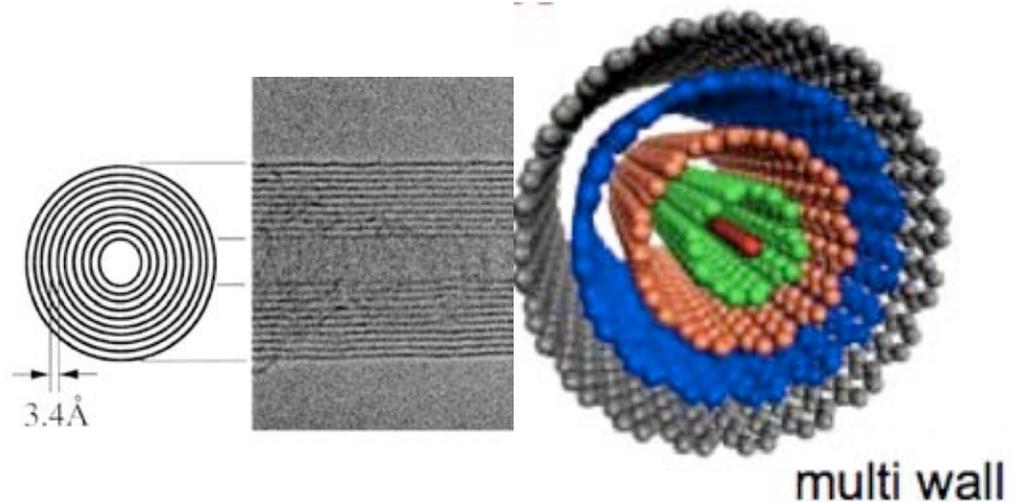
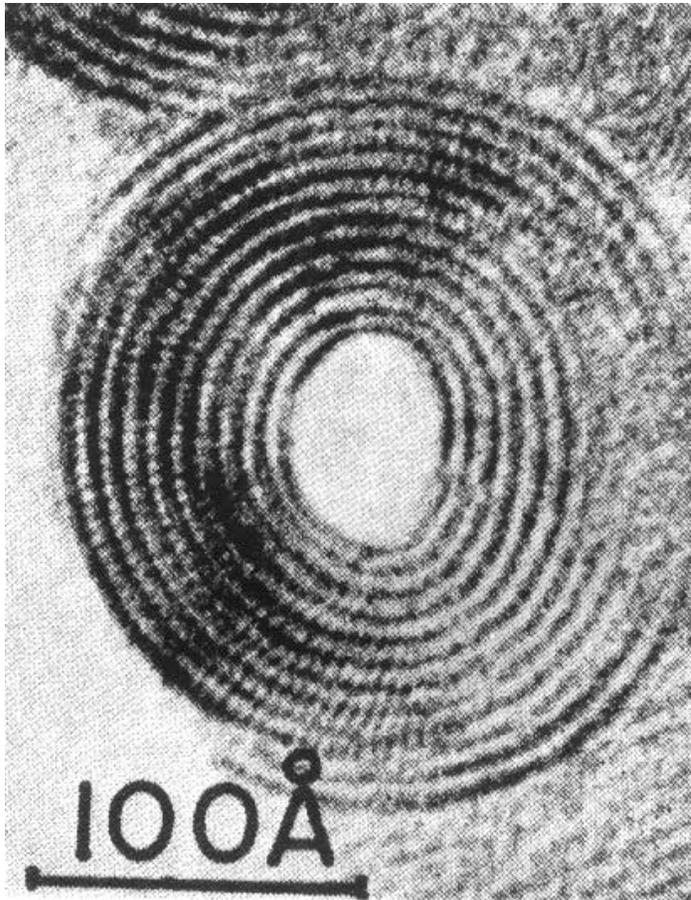
AND

- **...toxicity of metal contaminants**

AND

- **..toxicity related to electrical conductivity**

Carbon Nanotubes Look A Lot Like Chrysotile Asbestos



Two similar appearing **nanofibers**

- Chrysotile asbestos (left)
- Multiwall carbon nanotube (above)

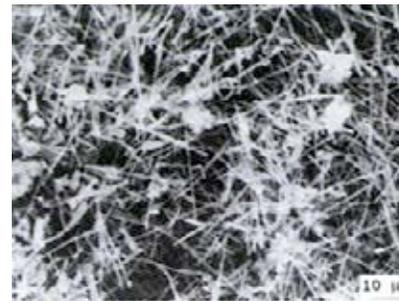
Similar toxicity?

www.gly.uga.edu/schroeder/geol6550/CM07.html

Fiber Toxicology



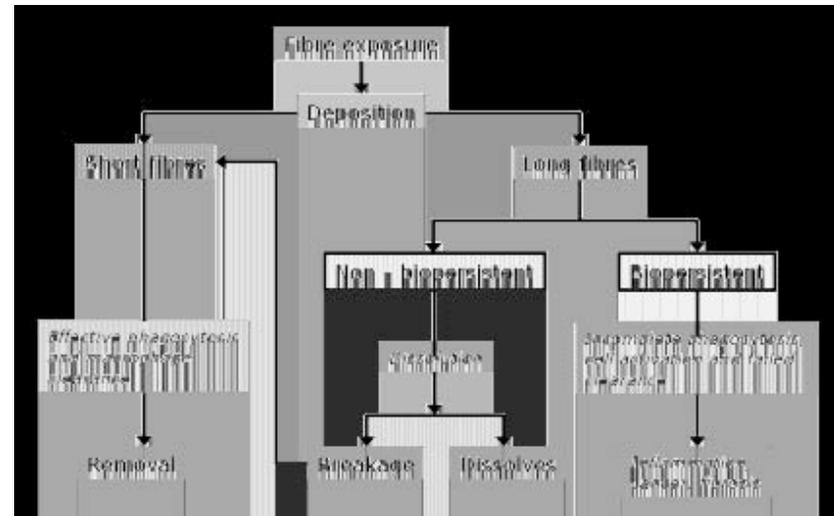
- **Many naturally occurring and man-made fibers can induce mesothelioma, lung cancer and/or pulmonary fibrosis:**
 - **Fibrous erionite & zeolite: High rate of mesothelioma in the Anatoly region of Turkey where they occur naturally—more potent than asbestos**
 - **Man made vitreous fibers**
Man made refractory ceramic fibers
 - **Silicon carbide whiskers: Similar potency to asbestos**
 - **Aluminum oxide, attapulgite, dawsonite, potassium titanate**



Fiber Toxicology



- **Key factors contributing to toxicity:**
 - Diameter < 1000 nm
 - Length $> 5,000$ nm:
 - High biopersistence
 - Poor pulmonary clearance
- **Chemistry probably matters too a lesser degree**



The three D's: Dose, dimensions and durability!

Carbon Nanotubes



**What has been published about
carbon nanotube toxicity?**

Occupational Toxicology Testing Methods



- **Inhalation > Aspiration > Instillation > In vitro**
- **Chronic > Sub acute > Acute**

Selected Cell Culture Studies



Year	Author	Cell Type	Conclusions
2003	Shvedova	Human skin fibroblasts	Cell death, oxidative stress (free radicals, peroxidized biomolecules, later shown to be related to Fe contamination), CNTs not internalized by cells
2005	Ding (LBNL)	Human skin/lung fibroblasts	MWCNT>MWCNO dose-dependent cytotoxicity and apoptosis, induce genes indicative of a strong immune, stress and inflammatory response-may mimic response to viral particles, a few dozen MWCNT could kill a cell
2005	Cui	Human embryonic kidney	SWCNTs inhibit cell growth by inducing cell apoptosis and decreasing cellular adhesion ability
2005	Jia	Human lung macrophages	SWCNT> MWCNT10>quartz>> C60 Cytotoxicity
2005	Murr	Mouse lung macrophages	S/MWCNT "ropes" showed dose related cytotoxicity, more toxic than asbestos
2005	Fiorito	Mouse & Human macrophages	Graphite>>SWCNT & C60, highly purified CNT were not well taken up by macrophages and caused little toxicity, metals cause CNT toxicity
2006	Kagan	Human lung macrophages	Much of oxidative stress is related to iron contamination, macrophages do not effectively detect or engulf CNT
2006	Tian	Human fibroblasts	Surface area predicts cytotoxicity, SWCNT>MWCNT
2006	Pluscamp	Lung macrophage and epithelial cells	M/SWCNT, raw and purified, little acute cytotoxicity, but did detect buildup of ROS intracellularly and decreased mitochondrial function which they ascribed to metal contaminants
2006	Tian	Human fibroblasts	Surface area predicts cytotoxicity, SWCNT>Activated carbon>CB>MWCNT>graphite>Carbon onions. Refined SWCNT more toxic than unrefined SWCNT
2006	Bottini	Human T Lymphocytes	Oxidized MWCNT 10x more cytotoxic to T cells than unaltered material, CB almost nontoxic. 10 ⁶ CNT/cell = 1 ng/cell max safe level
2006	Magrez	Human lung tumor cells	CB>MWCNTs reduced cell viability. Oxidation increases toxicity substantially
2007	Dvoren	Human lung epitheliod	Cytotoxic only at highest doses, not taken up by macrophages
2007	Wick	Human mesothelioma	Nanoropes>asbestos>Dispersed CNT Cytotoxicity

Pulmonary Toxicity: All Published Instillation/Aspiration Studies up to Mid 2008



Year	Author	Species	Granuloma	Inflamm	Diffuse Fibrosis	Death	Other
2001	Huczko	Guinea pig	–	No	–	No	
2004	Warheit	Rat	Yes ^a	Yes ^b	No	Yes ^c	
2004	Lam	Rat	Yes	Yes	–	Yes ^c	
2005	Muller	Rat	Yes	Yes	Yes	Yes	Biopersistent
2005	Huczko (Grubek- Jaworska)	Guinea pig	Yes	Yes	Yes	No	Pneumonitis
2005	Shvedova	Mouse	Yes	Yes ^b	Yes ^d	No	Odx. Stress
2006	Mangum	Rats	Yes	No	Yes	No	
2006	Carrero Sanchez	Mice	Yes	Yes	–	Yes	
2007	Shevdova	Mice	Yes	Yes	Yes	Yes	Oxd. Stress, esp. w/o vit. E
2008	Han	Mice	–	Yes	–	–	

A = non-uniform, b = transient, c = by choking, d = progressive

Warheit, 2004



Conclusion:

“...the pulmonary toxicity study findings of multifocal granulomas that we have reported herein may not have physiological relevance and may be related to the instillation of a bolus of agglomerated nanotubes (i.e., nanoropes).”



- **Conclusion:**

—“*...if nanotubes reach the lungs, they are much more toxic than carbon black and can be more toxic than quartz...*”



Conclusion:

“...if workers are exposed to respirable SWCNT particles at the current PEL (for graphite particles) they may be at risk of developing some lung lesions.”



Conclusion:

“...if multiwalled carbon nanotubes reach the lung they are biopersistent...and induce lung inflammation and fibrosis.”

“...the precautionary principle should be applied and adequate industrial hygiene measures implemented”

Huczko, 2005



Conclusion: “...CNTs should be considered a serious occupational health hazard...”

Magnum, 2006



Conclusions:

“...SWCNTs do not cause lung inflammation and yet induce the formation of small, focal interstitial fibrotic lesions in the alveolar regions of the lungs of rats”

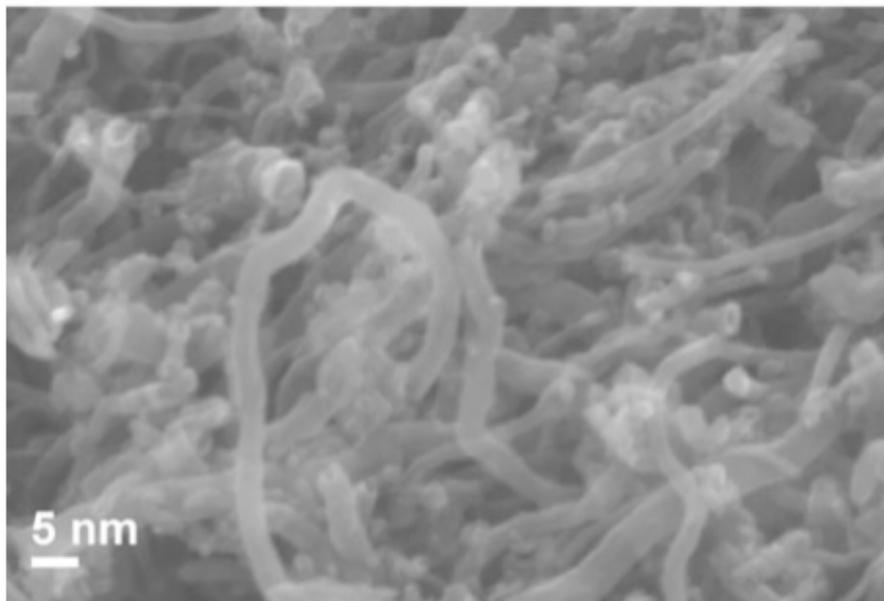
“...low levels of contaminating metals coupled with high surface area determine the toxicity and fibrogenic potential of SWCNT.”

First Published Inhalation Study-Mitchell

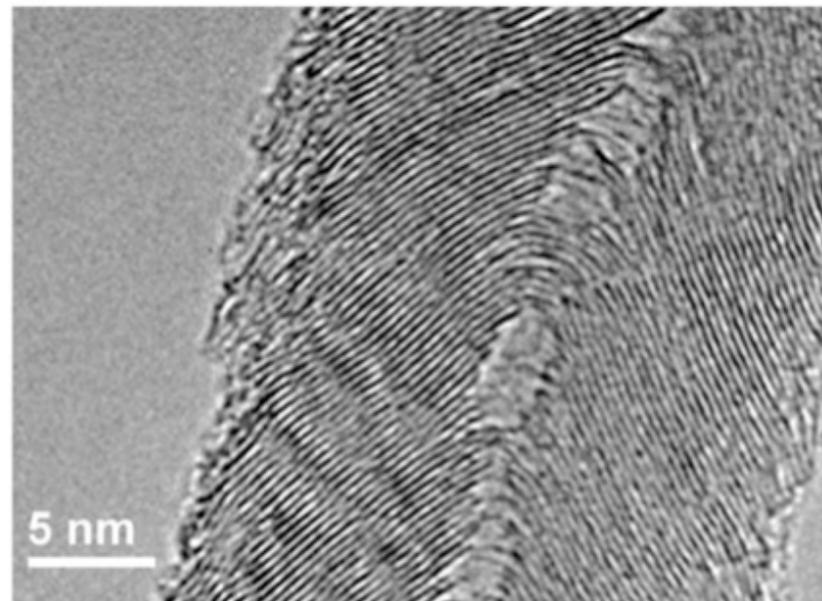


- Mitchell et al 2007: Representative “carbon nanotube” was in fact a carbon nanofiber, and the authors didn’t know the difference!
- 1 or 5 mg/m³ for up to 14 days
- **No inflammation or fibrosis or oxidative stress in lung**
- Suppression of T-lymphocyte function in spleen
- NIOSH writes this study off as a “mistake”

A



B



First NIOSH Inhalation Study



Am J Physiol Lung Cell Mol Physiol (July 25, 2008). doi:10.1152/ajplung.90287.2008

Submitted on April 23, 2008

Revised on June 24, 2008

Accepted on July 18, 2008

INHALATION VERSUS ASPIRATION OF SINGLE WALLED CARBON NANOTUBES IN C57BL/6 MICE: INFLAMMATION, FIBROSIS, OXIDATIVE STRESS AND MUTAGENESIS

Anna A. Shvedova^{1*}, Elena R. Kisin¹, Ashley R. Murray, Victor J Johnson², Olga Gorelik³, Sivaram Arepalli³, Ann F. Hubbs¹, Robert R. Mercer¹, Phouthone Keohavong⁴, Nancy Sussman⁴, J. Jin⁴, J. Yin⁴, Samuel Stone¹, Bean T Chen¹, Gregory Deye¹, Andrew Maynard⁵, Vincent Castranova¹, Paul A. Baron¹, and Valerian E Kagan⁴

¹ NIOSH

² National Institute for Occupational Safety and Health

³ NASA

⁴ University of Pittsburgh

⁵ Woodrow Wilson Center

First NIOSH Inhalation Studies



- **2-4x more lung inflammation than aspiration**
- **2-4x more diffuse fibrosis and granuloma formation**
- **Mutation of K-ras gene locus that is associated with cancer in these mice**

Mutation of Oncogene



- **Activated K-ras gene (63% vs 26%)**
- **Time-wise occurred at peak of inflammation**
- **Single point mutation that causes one amino acid substitution**
- **Previously implicated in lung cancer in mice exposed to chemical carcinogens**

Conclusion



- ***“Based on the outcomes of our inhalation study, it could be inferred that if workers were subjected to long-term exposures to respirable SWCNT at the current PEL for synthetic graphite, they would likely have increased risk for pulmonary changes.”***

Hot off the Presses from BASF



INHALATION TOXICITY OF MULTI-WALL CARBON NANOTUBES IN RATS EXPOSED FOR 3 MONTHS.

[Ma-Hock L](#), [Treumann S](#), [Strauss V](#), [Brill S](#), [Luizi F](#), [Mertler M](#), [Wiench K](#), [Gamer AO](#), [van Ravenzwaay B](#), [Landsiedel R](#).

Product Safety, BASF SE, 67056 Ludwigshafen, Germany.

Carbon nanotubes (CNT) are of great commercial interest. Theoretically, during processing and handling of CNT and in abrasion processes on composites containing CNT, inhalable CNT particles might be set free. For hazard assessment, we performed a 90-day inhalation toxicity study with a multi-wall CNT (MWCNT) material (Nanocyl NC 7000) according to OECD test guideline 413. Wistar rats were head-nose exposed for 6 hours/day, 5 days/week, 13 weeks, total 65 exposures, to MWCNT concentrations of 0 (control), 0.1, 0.5 or 2.5 mg/m³. Highly respirable dust aerosols were produced with a proprietary brush generator which neither damaged the tube structure nor increased reactive oxygen species on the surface. Inhalation exposure to MWCNT produced no systemic toxicity. However, increased lung weights, pronounced multifocal granulomatous inflammation, diffuse histiocytic and neutrophilic inflammation, and intra-alveolar lipoproteinosis were observed in lung and lung-associated lymph nodes at 0.5 and 2.5 mg/m³. These effects were accompanied by slight blood neutrophilia at 2.5 mg/m³. Incidence and severity of the effects were concentration-related. At 0.1 mg/m³, there was still minimal granulomatous inflammation in the lung and in lung-associated lymph nodes; a no observed effect concentration was therefore not established in this study. The test substance has low dust-forming potential, as demonstrated by dustiness measurements, but nonetheless strict industrial hygiene measures must be taken during handling and processing. Toxicity and dustiness data such as these can be used to compare different MWCNT materials and to select the material with the lowest risk potential for a given application.

PMID: 19584127 [PubMed - as supplied by publisher]

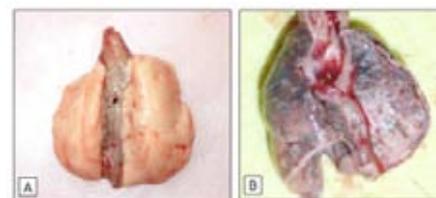


Figure 8. A) lung of a control animal B) lung of a high concentration animal with grey discoloration due to carbon nanotube deposition.

Publication of the study has sparked criticism from US NGO Environmental Defense Fund which notes that when BASF notified the product to the US Environmental Protection Agency last year, it claimed confidentiality over the identity of the product, even whether it was single or multi-walled, yet it is now prepared to discuss such details in a scientific journal.

Hot off the Presses from China



The acute pulmonary toxicity in mice induced by multiwall carbon nanotubes, benzene, and their combination.

[Li YS](#), [Li YF](#), [Li QN](#), [Li JG](#), [Li J](#), [Huang Q](#), [Li WX](#).

Biology Department, Life Science College, East China Normal University, Shanghai 200063, China.

Carbon nanotubes (CNTs) have been synthesized and produced on large scale for their wide application. They have high absorption ability to organic contaminants (such as benzene) and can form CNTs-benzene combination with benzene. In this article, the acute pulmonary toxicity, induced by multiwall carbon nanotubes (MWCNTs), benzene, and their combination, was studied by administrating the three test materials into mice lungs via intratracheal instillation. The biochemical parameters in bronchoalveolar lavage fluid (BALF) and pathological lesions in lungs were used as endpoints to evaluate the pulmonary toxicity of the three test materials at 3-day and 7-day postexposure, respectively. After the mice were intratracheally instilled with MWCNTs, benzene and MWCNTs-benzene combination at doses of 6.67 mg/kg, 2.67 mg/kg, and 9.34 mg/kg (containing 6.67 mg/kg MWCNTs and 2.67 mg/kg benzene), the total protein, alkaline phosphatase (ALP), acid phosphatase (ACP), and lactate dehydrogenase (LDH) in BALF and pathological lesions in lungs were examined. At 3-day postexposure, MWCNTs induced obvious pulmonary toxicity and benzene only induced slight pulmonary toxicity, whereas their combination induced very severe pulmonary toxicity. At 7-day postexposure, MWCNTs and benzene did not induce pulmonary toxicity individually, whereas their combination still induced severe pulmonary toxicity. These data indicated that, at the instilled doses in this experiment, the MWCNTs can alone induce acute pulmonary toxicity in mice and the benzene does not induce pulmonary toxicity, but the pulmonary toxicity of MWCNTs is enhanced after they form MWCNTs-benzene combination with low dose of benzene. The enhanced pulmonary toxicity may be due to the change of MWCNTs aggregation ability after benzene is adsorbed on them. (c) 2009 Wiley Periodicals, Inc. Environ Toxicol 2009.

PMID: 19526538 [PubMed - as supplied by publisher]

•Toxic synergy between nanotubes and benzene

Can Inhaled Fibers Migrate To the Pleura to Potentially Cause Mesothelioma?



 Department of Health and Human Services
Centers for Disease Control and Prevention

NIOSH Science Blog

Persistent Pulmonary Fibrosis, Migration to the Pleura, and Other Preliminary New Findings after Subchronic Exposure to Multi-Walled Carbon Nanotubes

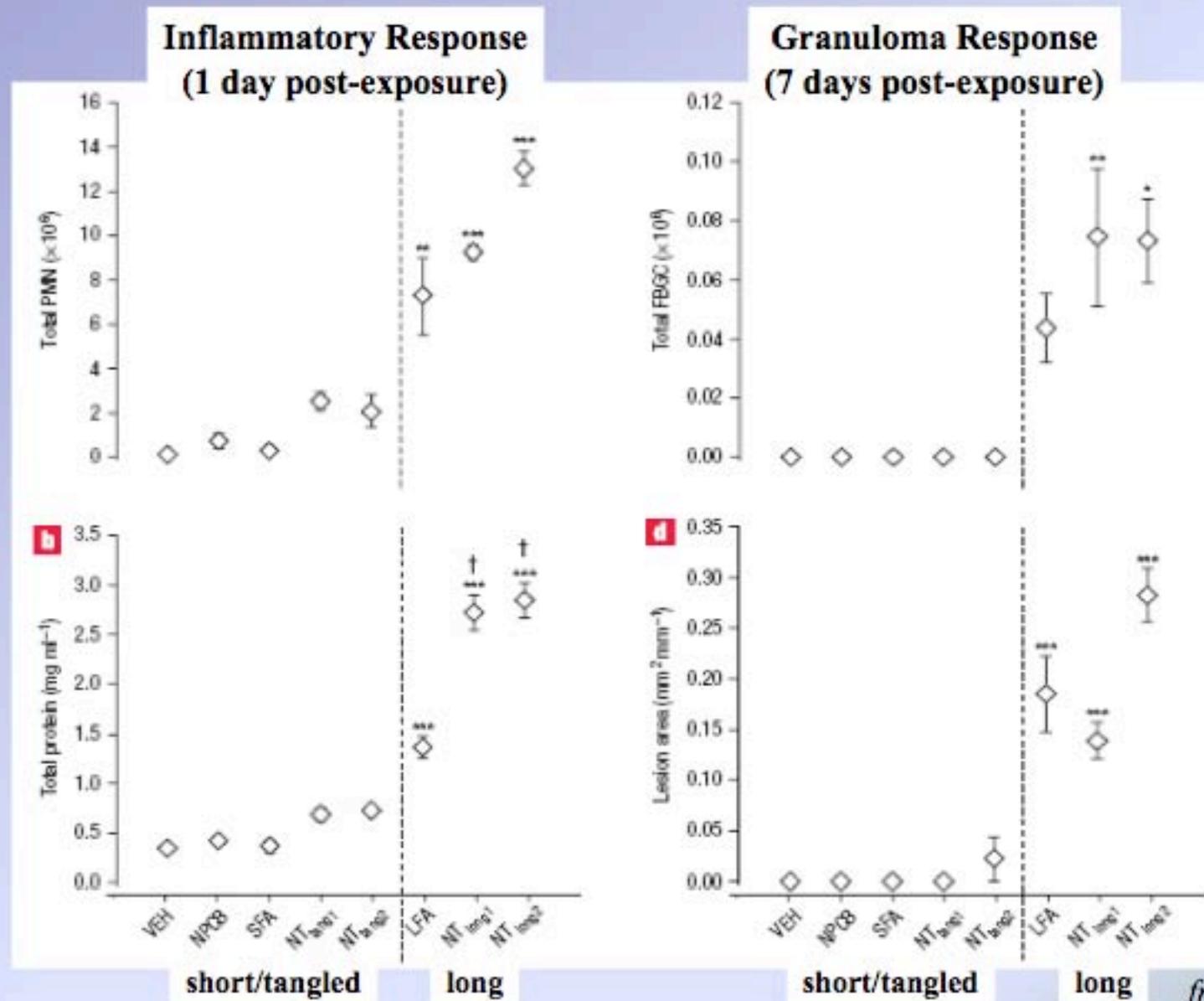
Multi-walled carbon nanotubes, known as MWCNTs for short, are a type of engineered nanomaterial that shows promise for various applications. These include the potential for creating stronger, more durable building materials; improving cancer therapies; creating more efficient means of energy generation, storage, and transmission; and speeding computer processes. However, as with other types of engineered nanomaterials, the potential occupational health implications of MWNCTs are not well understood at this emergent stage of the technology. A broad group of health and safety practitioners and business observers have agreed that research is vital for determining if MWCNTs pose a health risk for workers engaged in their production and industrial use, and for informing the responsible development of this technology. There is general agreement that this issue must be approached in a proactive manner with good research in order for society to benefit from the many promises this new class of materials has to offer.

NIOSH, 2009

Do MW-CNTs Behave Like Asbestos in *In Vivo* Studies?

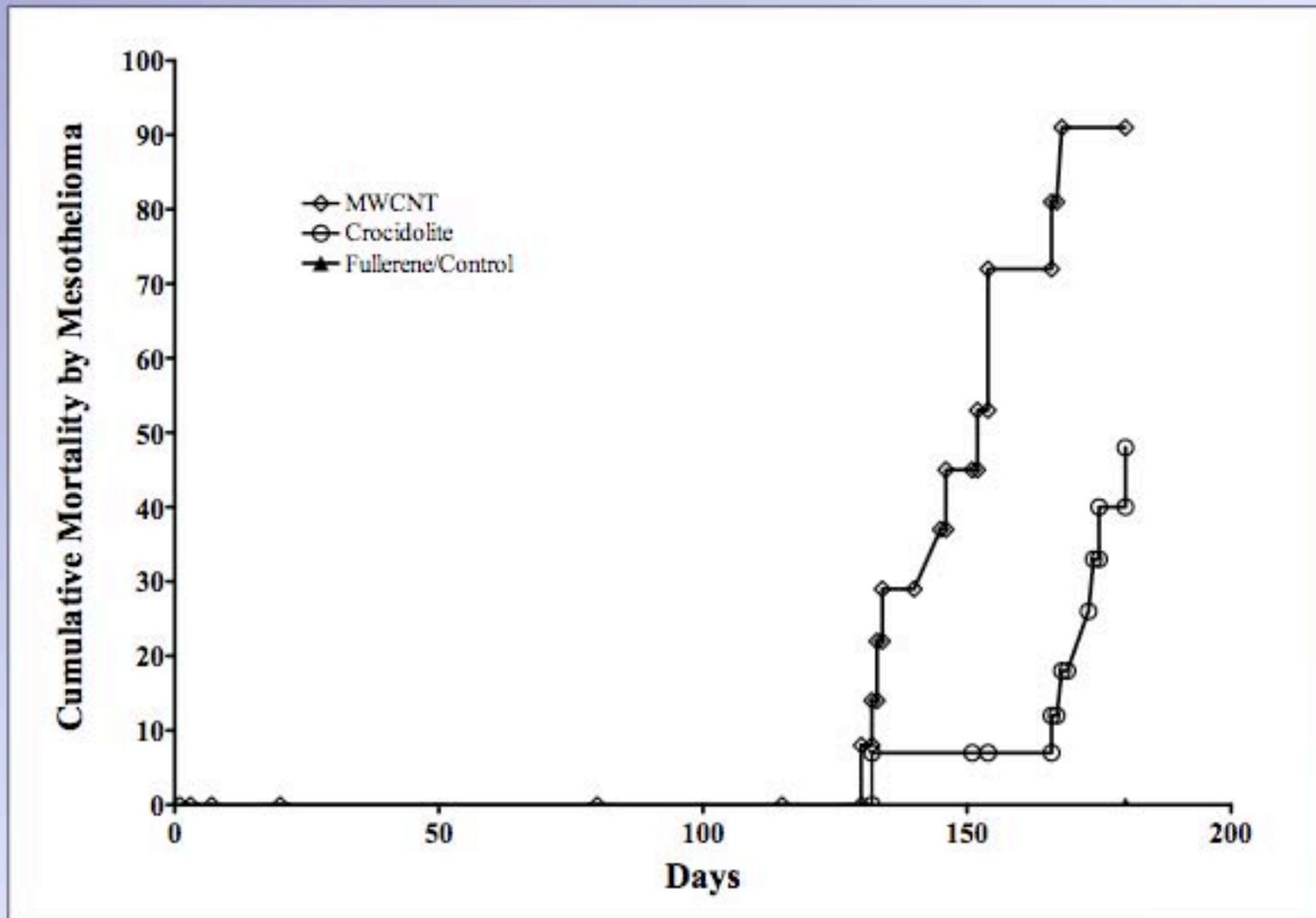
1. Poland et al., 2008 (*Nature Nanotechnol.*)
 - Intraperitoneal injections (50 μg) in mice;
 - Compared 4 CNT preparations to carbon black and short and long amosite fibers.
2. Takagi et al., 2008 (*J. Toxicol. Sci.*)
 - Intraperitoneal injections (3 mg) in p53^{+/-} mice;
 - Compared to crocidolite fibers and fullerene.

Do MW-CNTs Behave Like Asbestos?



from Poland et al., 2008

Do MW-CNTs Behave Like Asbestos in p53^{+/-} Mice?



Adapted from Takagi et al., 2008

Hot off the Presses



□ 1: [J Toxicol Sci.](#) 2009 Feb;34(1):65-76.

Induction of mesothelioma by a single intrascrotal administration of multi-wall carbon nanotube in intact male Fischer 344 rats.

[Sakamoto Y](#), [Nakae D](#), [Fukumori N](#), [Tayama K](#), [Maekawa A](#), [Imai K](#), [Hirose A](#), [Nishimura T](#), [Ohashi N](#), [Ogata A](#).

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The present study assessed a carcinogenic hazard of multi-wall carbon nanotube (MWCNT) in intact (not genetically modified) rodents. MWCNT (1 mg/kg body weight, 7 animals), crocidolite (2 mg/kg body weight, 10 animals) or vehicle (2% carboxymethyl cellulose, 5 animals) was administered to male Fischer 344 rats (12 weeks old) by a single intrascrotal injection. Rats were autopsied immediately after death, when becoming moribund or at the end of the maximal observation period scheduled to be 52 weeks. After 37-40 weeks, however, 6 MWCNT-treated animals died or became moribund due to intraperitoneally disseminated mesothelioma (6/7, 85.7%) with bloody ascites. Peritoneal mesothelium was generally hypertrophic, and numerous nodular or papillary lesions of mesothelioma and mesothelial hyperplasia were developed. While mesothelioid cells were predominant in relatively early stage tumors, advanced stage mesotheliomas were constituted by 2 portions occupied by mesothelioid cells on the surface and spindle-shaped sarcomatous cells in the depth. In the latter, the histological transition was apparently observed between these 2 portions. Mesotheliomas were invasive to adjacent organs and tissues, and frequently metastasized into the pleura. Only 1 rat survived for 52 weeks in the MWCNT-treated group, and similar findings except mesothelioma were observed. All 10 crocidolite-treated and 5 vehicle-treated rats survived for 52 weeks without any particular changes except deposition of asbestos in the former case. It is thus indicated that MWCNT possesses carcinogenicity causing mesothelioma at a high rate in intact male rats under the present experimental conditions. The present data identifies a carcinogenic hazard of MWCNT and will serve as one of the indispensable evidences to be used for the risk assessment crucial for not only protection and improvement of human health and welfare, but also safe and acceptable development and prevalence of this and similar upcoming materials.

PMID: 19182436 [PubMed - indexed for MEDLINE]

- **Again, more potent carcinogen than crocidolite!**

Really Hot off the Presses



1: [Toxicol Sci.](#) 2009 Aug;110(2):442-8. Epub 2009 May 8.

Absence of carcinogenic response to multiwall carbon nanotubes in a 2-year bioassay in the peritoneal cavity of the rat.

[Muller J](#), [Delos M](#), [Panin N](#), [Rabolli V](#), [Huaux F](#), [Lison D](#).

Industrial Toxicology and Occupational Medicine Unit, Catholic University of Louvain, 1200 Brussels, Belgium.

Toxicological investigations of carbon nanotubes have shown that they can induce pulmonary toxicity, and similarities with asbestos fibers have been suggested. We previously reported that multiwall carbon nanotubes (MWCNT) induced lung inflammation, granulomas and fibrotic reactions. The same MWCNT also caused mutations in epithelial cells in vitro and in vivo. These inflammatory and genotoxic activities were related to the presence of defects in the structure of the nanotubes. In view of the strong links between inflammation, mutations and cancer, these observations prompted us to explore the carcinogenic potential of these MWCNT in the peritoneal cavity of rats. The incidence of mesothelioma and other tumors was recorded in three groups of 50 male Wistar rats injected intraperitoneally with a single dose of MWCNT with defects (2 or 20 mg/animal) and MWCNT without defects (20 mg/animal). Two additional groups of 26 rats were used as positive (2 mg UICC crocidolite/animal) and vehicle controls. After 24 months, although crocidolite induced a clear carcinogenic response (34.6% animals with mesothelioma vs. 3.8% in vehicle controls), MWCNT with or without structural defects did not induce mesothelioma in this bioassay (4, 0, or 6%, respectively). The incidence of tumors other than mesothelioma was not significantly increased across the groups. The initial hypothesis of a contrasting carcinogenic activity between MWCNT with and without defects could not be verified in this bioassay. We discuss the possible reasons for this absence of carcinogenic response, including the length of the MWCNT tested (< 1 μm on average), the absence of a sustained inflammatory reaction to MWCNT, and the capacity of these MWCNT to quench free radicals.

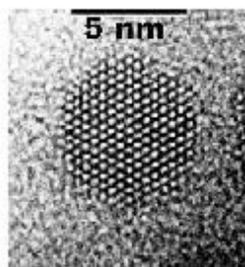
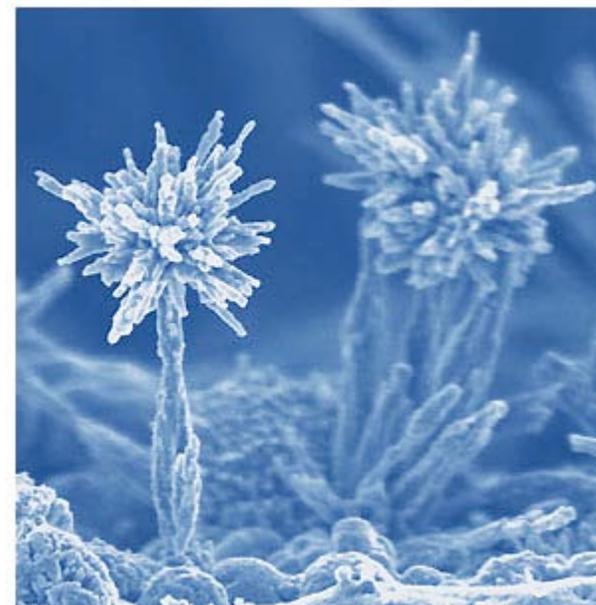
PMID: 19429663 [PubMed - in process]

- **Used fibers <1μm in length, result not surprising**

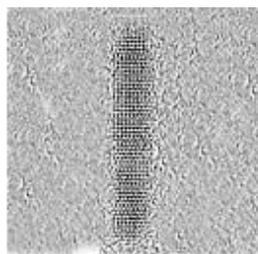
Toxicity: Unknown



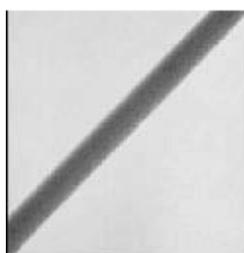
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Spheres



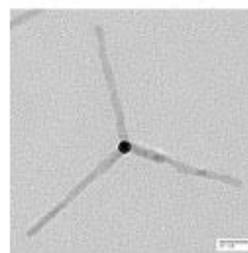
Rods



Wires



Ribbons



Tetrapods

- Chronic toxicity??
- Reproductive toxicity??
- Neurotoxicity??
- Hepatotoxicity??
- Endocrine disruption??

Questions?

