Nanotoxicology as a Predictive Science that can be explored by high content screening and the use of computer-assisted hazard ranking

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Proposal

Predictive Toxicological Paradigm to resolve the safety assessment of a large number of nanomaterials

In Vivo Adverse Outcomes

- Maximum of $10^2$ animals per experiment (weeks to months)
- Material physicochemical properties
  - mechanism of injury
  - toxicological pathway

Cellular or Bio-molecular Injury Endpoints

- Up to $10^5$ measurements per day

Validity of predictions

QSARs

Nel et al Science 2006.  
Huan Meng et al ACS Nano. 2009
Talk Outline

1. Why do we need a predictive science for hazard ranking and decision making?

2. What exactly are the linkages that connect the *in vitro* knowledge domain to *in vivo* hazard assessment?

3. What is the appropriate place for high content or high throughput screening and nano informatics in a predictive hazard platform?

4. What other benefits are there in using a predictive toxicology approach?
Prolific Growth of Nanotechnology

Source: Project on Emerging Nanotechnologies
Why do we need a Predictive Science for ENM hazard assessment?

1. Need for a hazard platform that keeps pace with the rate at which new ENMs are being introduced

2. It is impossible to use animal testing as the primary platform for ENM hazard assessment

3. Need for an *in vitro* property-activity approach that utilizes high-volume data collection to prioritize and speed up *in vivo* decision-making

4. Need for a robust scientific platform to link *in vitro* to toxicological relevant *in vivo* outcomes (will propose mechanistic toxicological pathways)
A large number of material properties and bio-interfaces generate an endless number of possibilities at the nano-bio interface.

Different sizes/shapes/aspect ratios
States of agglomeration
Media interactions
etc etc

Cell membrane
Proteins in the medium, cells et
DNA and nucleus
Cell uptake (endocytosis/phagocytosis etc)
Subcellular localization/organellar interactions
Mitochondrial functions/ATP production
Bio-accumulation/biopersistence
etc etc

Nel et Nature Mat. 2009
It’s All So Complex but ..

We can track NP and use their properties.

We can make model libraries of materials.

We can use high throughput approaches.

We can use computational power to enhance and speed up decision-making.

We can use property-activity relationships to make safer materials or prevent exposure to materials with hazardous properties.

• Provide wide coverage of potential toxicants
• Use a robust scientific base for testing (instead of a descriptive approach in whole animals)
• Comprehensive array of predictive in vitro tests that utilize toxicity pathways and mechanisms
• High content or high throughput screening to facilitate testing of large batches of materials
• In vitro hazard to be confirmed in vivo

http://www.nap.edu/catalog.php?record_id=11970
NP toxicological considerations covered in this talk

1. Predictive pulmonary toxicity
2. Predictive environmental toxicology, e.g., zebrafish
Predictive toxicology in air pollution research
Oxidative Stress as a Predictive Toxicological Paradigm: Real-life Proof that in vitro assessment of oxidant potential is linked to the exaggerated cardiovascular effects of ultrafine particles.

Generate Oxidant Injury in the Lung and Cardiovascular system (the lung signal is from an oxidative stress gene that is turned in a live animal exposed on an LA freeway).

Increased rate of atherosclerosis in UFP compared to PM2.5 exposures.
Oxidative Stress as a Predictive Toxicological Paradigm: Use of the mouse asthma model to demonstrate that ultrafine oxidant potential is linked to allergic sensitization.

Dendritic cells

↓ Th1

↑ Th2

Chan et al. JACI. 2006
Li et al. J Immunol
Li et al. EHP. 2009
A proposed paradigm for ENM pulmonary toxicity evaluation: Concept of NP Surface Reactivity

**Surface Area Dose**
- **Very High Surface Reactivity:**
  - Crystalline Si (quartz)
  - Ni
  - Co

- **High Surface Reactivity:**
  - Cu
  - High cationic charge
  - ZnO

- **Low Surface Reactivity:**
  - TiO$_2$
  - Au
  - Carbon black
  - Amorphous Si
  - Polystyrene

**Pulmonary Inflammation**
Example: When Using IL-8 production in a bronchial epithelial cell line to discern between the inflammation potential of High versus Low Reactive Surface Area NP in rats

![Graph showing In Vitro and In Vivo data]

- **In Vitro**
  - IL-8 (pg/ml) vs. Surface Area (cm²)
- **In Vivo**
  - Neutrophil content of BALF (x10⁵) vs. Surface Area (cm²)

Quartz, Co, Ni

Adapted from Duffin et al Inhal Tox 2007
Surface Reactivity Paradigms

A. Cationic toxicity
Lysosomal, surface membrane

B. Membrane
Lysis, e.g., charge

C. Redox active

D. Immune danger signals, inflammazones
e.g., dendritic cells

E. Dissolution and ion release
e.g., ZnO

Nel et al. Science. 2006
Metal Fume Fever: Metal oxide toxicity

Welders exposed to ZnO, other metal oxides: Cu, Mg, Sn, or Cd

3-10 hrs post-exposure: flu-like illness, fever, malaise, chills, dry cough, shortness of breath

BAL cytokines: TNF$_\alpha$, IL-6, IL-8, MIP

Pathophysiology: marked increases in lung PMLs 20–24 hr after exposure

Resolves 24–48 hr after onset, no structural damage

Short-term tolerance: asymptomatic with repeated exposure
**ZnO dissolution chemistry and cellular toxicity**

Xia et al
ACS Nano

**Superoxide**

- % MitoSOX Red$^+$ Cells
- Time (h)

- *ZnO
- *CeO$_2$ TiO$_2$

**ZnO**

- *

**MMP**

- % JC-1 low Cells
- Time (h)

- *ZnO
- *CeO$_2$
- *TiO$_2$

**PI uptake**

- % PI+ Cells (M1)
- Time (h)

- *ZnO
- *CeO$_2$ TiO$_2$

**TNF-α**

- TNF-α (pg/ml)
- Time (h)

- *ZnO
- *CeO$_2$ TiO$_2$

**Overlay**

LAMP-1

Newport Green

**Total [Zn]**

- [Zn] (µM)

- BEGM
- CDMEM
- H$_2$O
Mouse studies showing *in vivo* linkage to cellular effects
Microarray analysis of whole blood total RNA in boilermakers before and after occupational exposure to metal fumes

Genes with altered expression were clustered in biologic groupings that reflect induction of:

- inflammatory responses: esp IL-8
- oxidative stress
- signal transduction
- programmed cell death

Predictive approach to environmental hazard assessment

Prioritize *in vivo* testing at increasing trophic levels

100’s/year 1000’s/year 10,000’s/day 100,000’s/day

High Throughput Bacterial, Cellular, Yeast, Embryo or Molecular Screening
Use of a Zebra fish model to perform Predictive Environmental Toxicology

Nanoparticles in working concentrations

Observe and score for mortality rate, hatching rate, morphology and physiology

David Schoenfeld, Tian Xia, Saji George, Ivy Ji, Shou Lin, Andre Nel
Comparison three MeO in vitro

Macrophage line

Superoxide

Epithelial line

Superoxide

Xia et al. ACS Nano. 2008
Dose- and time- dependent rapid throughput cytotoxicity assay in BEAS-2B cells

- ZnO showed dose and time dependent increase in all parameters of toxicity
- TiO$_2$ and CeO$_2$ showed no cytotoxicity

Comparing the toxicity of the MeO library in Zebrafish

- Control
- CeO\textsubscript{2}
- TiO\textsubscript{2}
- ZnO

**Survival rate**

- % of embryos

- n = 24. * p<0.001

**Hatching rate**

- With Chorion
- Without Chorion

- ZnO-Unhatched alive
- ZnO-Unhatched dead

- 25 ppm
- 50 ppm

- Control
- ZnCl\textsubscript{2}
- ZnO
What are the key ingredients for establishing a predictive science at the nano-bio interface?

1. The development of appropriate cellular and bio-molecular assays that can be used for predicting ENM hazard in intact animals

2. Development of compositional and combinatorial ENM libraries that can be used to explore property-activity relationships

4. Ability to perform high throughput screening to speed up knowledge generation

5. Computational analysis and nano-bioinformatics to deal with high volume data sets and ability to make predictions
The use of Compositional and Property-based Nanomaterial Libraries to make discoveries at the nano-bio interface

Oxides
1st: TiO$_2$, CeO$_2$, ZnO

Silica
Amorphous, Crystalline, Mesoporous

Compositional:

Metals
Au, Ag, Pt, Co

Carbon Nanotubes
SWCNT, MWCNT

Clays
Montmorillonite, Attapulgite, Kaolin

Size
Shape
Composition

Zhaoxia Ji and Jeffrey Zink

Group Leader: Jeff Zink (UCLA)
Property Variations in Combinatorial Libraries

- Angle of curvature
  - Surface area
- Size
  - Shape
  - Porosity
- Aspect ratio
- Corrugation effects
- Bandgap
- Solubility
- Metal doping
- Photo-activation
- Redox activity
- Defects
- Surface reconstruction
- Crystallinity and phase transition
- Electronic states
- Surface functionalization
- Cationic groups
- Disolution
- Surface functionalization
- Ligands
- Dangling bonds
- Size
- Shape
- Porosity
- Angle of curvature
- Aspect ratio
- Corrugation effects
- Bandgap
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- Cationic groups
- Disolution
- Surface functionalization
- Ligands
- Dangling bonds
Design of an Fe-doped ZnO library

High throughput toxicity screening

Mechanism of ZnO nanoparticle toxicity

Proposed safe material design


Nuclear area
Loss of MMP
Cellular Ca
PI Uptake

ZnO NPs
ZnO dissolution
[Ca^{2+}]_{i}
ROS
Membrane permeability
PI uptake
Cytotoxicity

Iron doped ZnO NPs
Reduced ZnO dissolution
Reduced cytotoxicity
ZnO-based Composition Library to Study the Role of Dissolution Chemistry in Toxicity

Cellular, bacterial, organism, animal toxicity screening, fate and transport

Lutz Mädler, Suman Pokhrel, Bremen
Iron doping alters the matrix and yields slower dissolving ZnO NP

Particles synthesized by Lutz Maedler, Germany

Low TiO$_2$ toxicity in Marine Phytoplankton under non-UV conditions changes under UV conditions

**Graphs:**
- **Left graph:** Growth rate (y-axis) vs. TiO$_2$ NP ug L$^{-1}$ (x-axis) with a trend line indicating a decrease in growth rate as TiO$_2$ NP concentration increases. The p-value is 0.2.
- **Right graph:** Growth rate (y-axis) vs. TiO$_2$ NPs µg L$^{-1}$ (x-axis) showing distinct points for high UV and UV blocked conditions. The points for high UV conditions are higher than those for UV blocked conditions.

**Image:**
- A close-up image of Thalassiosira pseudonana, a type of marine phytoplankton.

**References:**
- IRG 3-1: Lenihan & Miller
To study photoactivation by TiO$_2$ mechanistically it is necessary to develop an ENM library that can be used under longer wavelenght conditions: bandgap tuning by Fe doping.
Construction of a cationic MSNP library by coating with PEI

Xia et al. ACS Nano. 2009
Shape and Aspect Ratio Property Library shows that aspect ratio has a profound effect in active uptake tied to a specific Cellar activation mechanism.
What linkages can be used for high content data generation to prioritize in vivo assessment?

Bio-Molecules
  • Genes
  • Proteins, etc

Cellular injury responses
  • Non-lethal
  • Lethal

Biological pathways
  • Toxicological
  • Signaling
  • Death pathways

Single cell or simpler life forms

Animal pathology, disease
  • Embryos
  • Vertebrates
  • Mammalian

Human health impact

Impact on higher life forms, predators, populations and ecosystems
# Robust Screening Platform: Mechanistic Cellular Injury Pathways

<table>
<thead>
<tr>
<th>Toxicological Pathway</th>
<th>Example Nanomaterials</th>
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<tbody>
<tr>
<td>Membrane damage/leakage</td>
<td>Cationic NPs</td>
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<tr>
<td>DNA cleavage/mutation</td>
<td>Nano-Ag</td>
</tr>
<tr>
<td>Mitochondrial damage &amp; apoptosis</td>
<td>ZnO, cationic NPs</td>
</tr>
<tr>
<td>Lysosomal damage: proton sponge effect frustrated phagocytosis</td>
<td>Cationic NPs, CNTs</td>
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<tr>
<td>Fibrogenesis and tissue remodeling</td>
<td>CNTs</td>
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<tr>
<td>Blood platelet, vascular endothelial &amp; clotting abnormalities</td>
<td>SiO$_2$</td>
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<tr>
<td>Signaling cascades</td>
<td>Metal oxide NPs, CNTs</td>
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<tr>
<td>Inflammation, gene expression, survival</td>
<td>CNTs, metal oxide NPs, cationic NPs</td>
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<tr>
<td>Oxidative stress injury</td>
<td></td>
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</tbody>
</table>

_Huan Meng et al ACS Nano. 2009_
Nanomaterial Mechanisms for Oxygen Radical production

- Excited state Electron donor active groups
- Semiconductor properties
- Electron hole pairs
- Dissolution
- Release of ions
- Redox cycling and catalytic chemistry

UV

H₂O

O₂

OH·

TiO₂

H₂O₂

Fe²⁺

OH·

Fenton chemistry

O₂

Q

TIO₂

Fullerene

Metal oxide

O₂⁻

Redox cycling organics

Q⁻

ZnO

CdSe

Ambient UFP Metal NP Carbon NT

Adapted Nel et al Science 2006.
The Hierarchical Oxidative Stress Model

Response pathways:
- Normal
- Anti-oxidant defense
- Inflammation
- Cytotoxicity

Signaling pathway:
- Nrf-2
- MAP Kinase NF-κB cascade
- Mitochondrial PT pore

Genetic response:
- Anti-oxidant response element
- AP-1 NF-κB
- N/A

Outcome:
- Phase II enzymes
- Cytokines Chemokines
- Apoptosis

Nel et al. Science, 311, 622-627, 2006
In vitro comparison of a panel of nanoparticles based on the hierarchical oxidative stress paradigm

Epithelial
Endothelial
Macrophage
Kidney
Liver
Neuronal
etc

Abiotic
Biotic

Tier 1
Phase 2 anti-ox enzymes
HO-1
GSH

Tier 2
Inflammation
cytokines
chemokines

Tier 3
Mitochondria
MMP
ATP
ROS
[Ca^{2+}]_{m}

Cell death
caspase activation
PI uptake
MTS assay

It is possible to do profiling:

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<th>ROS</th>
<th>Tier 1</th>
<th>Tier 2</th>
<th>Tier 3</th>
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<tr>
<td>Particle 6</td>
<td>cell</td>
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</tbody>
</table>

Xia et al. Nano Letters. 2006
Meng H et al. ACS Nano 2009

CB
Polystyrene
TiO_2
Fullerol
UFP
NH2-Ps
e tc
Interconnected final common pathways of NM injury to screen for lethal and sublethal responses

George et al. ACS Nano. 2010
Establishment of a Multi-parametric Assay

- Metal impurities
- Organic adsorbants
- Dissolution metal ion release
- Redox active surfaces
- Photoactivation electron-hole pairs

Redox cycling chemistry

ROS

- Lysosome (↓pH)
- Toxic ions
- Mitochondrion

- [Ca^{2+}]_i
- Membrane permeability
- Cell viability
- MMP Caspase 9 ATP
- Membrane association
- Cationic particles Ambient UFP
- O_{2}^{-}

George et al. ACS Nano. 2010
Multi-parametric Oxidative stress High Throughput Screen

- ROS generation
- Mitochondrial membrane depolarization
- Cytotoxicity and PI uptake
- Intracellular Ca flux
- Cell localization / nucleus

Cells
Bacteria
Yeast
Embryos

Group Leader: Ken Bradley (UCLA)
MSSR Director: Robert Damoiseaux
Plate layout for each cell type

16 rows
24 columns

Plate#1: Hoechst 33342 + MitoSox Red
Plate#2: Hoechst 33342 + JC1
Plate#3: Hoechst 33342 + Fluo-4 + PI
<table>
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<tr>
<th>NMs</th>
<th>Size (nm)</th>
<th>Zeta potential (mV)</th>
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<td>Qdot-T</td>
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<td>48.5</td>
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<tr>
<td>ZnO</td>
<td>130.5</td>
<td>24.23</td>
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HTS requires high throughput methods to assess particle suspension and stability
High Throughput DLS of the Kinetics of NP agglomeration in mammalian tissue culture media

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<th>NPs</th>
<th>Con (µg/mL)</th>
<th>CDMEM</th>
<th>BEGM+2mg/mLBSA</th>
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Heat Map of the multi-parametric data (z-score transformation)

-2
-1
0
1
2

Time (hrs)
1...7 24

Macrophage

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<th>PI</th>
<th>iCa</th>
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Bronchial Epithelial

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<th>PI</th>
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Ag
Au
Pt
Al₂O₃
SiO₂
Qdot-T
ZnO

Dose (µg/mL)
0.325
12.5
50
100
Comparison of multi-parametric data to individual responses assessment

ATP measurement

Cell viability by MTS assay

Macrophage

Bronchial Epithelial

Qdot - T
Advantage of Multi-parametric testing: Revealing hidden relationships

SOM clusters emerging from the stacking of component planes

Component planes

2D visualization of the relationship between all cytotoxic parameters, all doses, time points and for all particles
SOM defined by similarity in cytotoxic response profiling for the entire data set
SOM defined by clustering of the biological response characteristics
Activity-Activity Relationships

**RAW 264.7 cells**
- ROS generation
- Mitochondria depolarization
- Intracellular Ca$^{2+}$ flux

**BEAS-2B cells**
- ROS generation
- Mitochondria depolarization
- Intracellular Ca$^{2+}$ flux
- Cell Membrane Damage

Correlation threshold: $|r|=0.5$

Effects after long exposure time: 24h
Pearson correlation for each cytotoxicity parameter was calculated from the robust z-score value.
High Throughput DLS of the Kinetics of NP agglomeration in Holtfreter’s medium

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</table>

Colors: Green = 100nm, Yellow = 500nm, Red = 1000nm
Correlation of HTS results to toxicity screening in zebrafish

- NPs (15ug/mL)

**Survival**

<table>
<thead>
<tr>
<th>Ranking</th>
<th>NPs</th>
<th>Morphological defects</th>
<th>Physiological defects</th>
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</thead>
<tbody>
<tr>
<td>0. No morphological or physiological defects</td>
<td>Au, Al2O3, Fe3O4, SiO2</td>
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<td>-</td>
</tr>
<tr>
<td>1. Single morphological or physiological defect</td>
<td>-</td>
<td>-</td>
<td>Low heart beat</td>
</tr>
<tr>
<td>2. Multiple morphological and physiological defects</td>
<td>Pt</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3. Severe multiple morphological and physiological defects</td>
<td>Ag</td>
<td>High mortality and reduced hatching rate and low heart beat</td>
<td></td>
</tr>
<tr>
<td>4. Embryo do not survive</td>
<td>ZnO, QD</td>
<td>Embryos do not survive or fail to hatch</td>
<td></td>
</tr>
</tbody>
</table>

* p<0.05

David Schoenfeld, Tian Xia, Saji George, Shou Lin, Andre Nel
Use of a Predictive Scientific Approach towards Safe design of ZnO

High throughput toxicity screening

Mechanism of ZnO nanoparticle toxicity

Proposed safe material design

- Nuclear area
- Loss of MMP
- Cellular Ca
- PI Uptake

ZnO NPs
- ROS
- [Ca^{2+}]_i
- Zn^{2+}
- Membrane permeability
- PI uptake
- Cytotoxicity
- Reduced ZnO dissolution
- Reduced cytotoxicity

Iron doped ZnO NPs

Doped ZnO Nanoparticles are less toxic in HTS analysis

![Graphs showing the percentage of positive cells for Macrophage and Bronchial epithelial cells with different iron oxide (Fe₃O₄) doping levels.](image)

Doped ZnO Nanoparticles are less toxic in Zebrafish Embryo Hatching Experiments

* Indicate p<0.05 from 0 ug/mL
Doped ZnO Nanoparticles are less toxic in pulmonary toxicity in mice

Murine IL-6 protein

Murine MCP-1 protein
Construction of a cationic MSNP library by coating with PEI

Xia et al. ACS Nano. 2009
Reduced polymer length, low toxicity MSNPs have high uptake in cancer cells

Fold ↑ cell fluorescence

Xia et al. ACS Nano. 2009
Reduced polymer length MSNPs allow siRNA Attachment but keep the pores available for doxirubicin loading.

Huan Meng et al. ACS Nano. 2010
Co-delivery of Pgp siRNA with Dox can Overcome Dox resistance in a Squamous carcinoma cell line

Huan Meng et al. ACS Nano. 2010
Differential toxicity of cationic NP depending on cellular differentiation

MSNP-PEI 25 kD
MSNP-PEI 10 kD
MSNP-PEI 1.8 kD
MSNP-PEI 1.2 kD
MSNP-PEI 0.8 kD
MSNP-PEI 0.6 kD
MSNP

1h  6h  Undifferentiated  Differentiated

Undifferentiated

Differentiated

200 µg/mL
0.2 µg/mL
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- Ning Li

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