A Rapid In Vivo System for Defining Biological Responses to Nanomaterials

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The Opportunities

Proactively guide the development of inherently safer nanomaterials

- Identify the physicochemical properties that drive behaviors – take a global view

- Think nanoscience

- Develop predictive “behavioral models” from experimental data.
Nanoparticles have widely tunable properties - the key is to enhance performance and safety at the same time.

Structure/Property Relationships:
Physicochemical properties and biological responses
The Nano Challenge

• Much in common with the small molecule challenge
• One material at a time approach will ultimately fail
• Generalizations cannot be made…yet
• We need (MUCH) more data
• We need paradigm shift in how we assess hazard
• Very little of this early data will be directly used for risk assessment.
How a material “behaves” absolutely depends on its physical properties

Agglomeration
Dissolution
Environmental Interactions
Environmental Fate
Biological Interactions
Biological Fate
Biological Responses

Goal is to predict these behaviors from inherent properties
Biology is a System that Responds

Exposure ↓
Tissue Dose ↓
Biologic Interaction ↓
Perturbation ↓

Low Dose

Normal Biologic Function

Biologic Inputs

Adapted from the National Academy
Toxicity Testing for the 21st Century
Biology is a System that Responds

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Entry Point = Toxicity Pathway

- Exposure
- Tissue Dose
- Biologic Interaction
- Perturbation

Biologic Inputs

Adaptive Stress Responses

Early Cellular Changes

Cell Injury

Normal Biologic Function

Morbidity and Mortality

Higher yet

Adapted from the National Academy
Toxicity Testing for the 21st Century
Toxicity Pathways

Toxicity Pathway: A cellular response pathway that, when sufficiently perturbed, is expected to result in an adverse effect.

• We need to identify these toxicity pathways

• Determine if NP perturb them
Nanomaterial Biological Assessments Platforms

• *In vitro*
  – Continuous cell culture system
  – Primary cell culture system

• *In vivo*
  – Whole animal studies
  – Rodents- slow and expensive
  – Zebrafish
  – Flies and worms – non vertebrates
Cell-Based Approaches

- Advantages - quick, easy and cheap

Response

Proliferation
Cell death
Metabolism
Gene expression
Phenotypic change

“There are blind spots”
What blind spots?

• Different cell-cell interactions cannot be evaluated
• Indirect effects cannot be evaluated
• Cells in culture can only respond using their unique repertoire of expressed gene products – limited potential targets
• Practical problem…what cells do you choose?
• Tremendous potential for missed data
• In vivo models can offer complementary data…….
Example

Cultured endothelial cells

Exposure and collect “omics data”

Hundreds of gene expression changes

Are these gene expression changes related to an adverse outcome? Do they represent an adaptive response?

What decisions can be made based solely on this information?
Systems Biological Approach - early embryonic development -

Why?

• Generally more responsive to insult... because
  
  Most dynamic life stage...and the full signaling repertoire is expressed and active, therefore fewer blind spots.. Highest potential to detect interactions

• If a chemical or nanomaterial is developmentally toxic it must influence the activity of a molecular pathway or process.. i.e. hit or influence a “Toxicity Pathway”

• Use the biological response to identify the “Toxicity Pathway”
Why We Use Zebrafish

• Share many developmental, anatomical, and physiological characteristics with mammals

• Molecular signaling is conserved across species

• Technical advantages of cell culture - power of in vivo

• Amenable to rapid whole animal mechanistic evaluations

• Focus on responses, then identity the “Toxicity Pathway” underlying it - immediately relevant
Development Stages of Assessments

1. 25hr
2. 6 hr
3. 19 hr
4. 24 hr
5. 48 hr
6. 120 hr
7. 4 hr

3 min

Zebrafish.mov
Assessing Biological - Nanomaterials Interactions and responses

**Tier 1: Toxicity Screening**
- Toxicity testing whole organisms
  - *In vivo* - zebrafish

**Tier 2: Cellular Targets and Distribution**
- Defined *in vivo*
  - Fluorescent nanomaterials
  - Targeted assays

**Tier 3: Molecular Expression**
- Genomic Responses
  - Whole animal gene expression profiles

Define Structure Activity Relationships
Toxicity Testing (First Steps)

A large adult colony is required to support testing laboratory

Remove Chorions

1 Embryo/well

Test Materials

Screening for responses 1-5 days
Alternate Exposure Route - Microinjection

1 cell stage

24 hpf
Consider Startpoints – Not Endpoints

- Signaling pathways and molecular events are conserved
- ...But fish are not rodents or humans
- Consequences of disrupted signaling often species specific
- ........the mechanism by which a “target” is hit is likely conserved, but the consequence of the “hit” may be distinct
Example of Toxicity Endpoints

TCDD
- Shortened Snout
- Pericardial EDEMA
- Head EDEMA
- Yolk Sac EDEMA
- Uninflated Gas Bladder

CONTROL
- Gas Bladder
Early Life Stage Responses to Ethanol
Interpreting Common Endpoints

Common endpoints revealed by chemical, nanomaterial and genetic screening

- Pericardial edema
- Yolk sac edema
- Reduced growth
- Bent body axis
- Lack of swim bladder inflation
- Mortality

Common nonspecific
AHR Signaling Pathway

TCDD

TCDD-Responsive Genes

Nucleus

Cytoplasm

Increased expression
Xenobiotic metabolizing enzymes
Toxicity?

AhR1b

AhR2 + AhR1a

AhR

AhR2

AIP

hsp 90

TCDD

mRNAs

ARNT

ARNT1

DRE

2a, 2b, 2c

Increased expression
Xenobiotic metabolizing enzymes
Toxicity?
Assess Gene Functions - *in vivo*

Morpholino Gene Repression – General approach

Translational Repression - Protein levels reduced
Assess Gene Functions - *in vivo*

Morpholino Gene Repression – General approach

Translational Repression- Protein levels reduced
Assess Gene Functions - *in vivo*

Morpholino Gene Repression – General approach

Translational Repression - Protein levels reduced

Since genome is sequenced
any gene can be targeted in days!

Morpholino knock-down

A

B

C

3 hpf
ARNT1 and AHR2 are Necessary for TCDD Toxicity
Repression of ARNT1 OR AHR2 Makes Fish Non-responsive to TCDD

- ARNT1
- AHR2
- TCDD
- DRE

TCDD-Responsive Genes

Mortality

No Toxic Response

No Toxic Response
Tier 1 continued

Early Behavioral Assessments
Spontaneous Behavior
Probing the CNS.......
Touch Responses

Normal

Twitch

Spasm

Non-responsive
Nanoparticles Assessed - to Date

Over 200 fully evaluated through tier 1.

$C_{60}$, $C_{60}(OH)_{24}$, $C_{70}$, SWCNT, DWCNT, dendrimers, metal oxides, Q-dots, gold nanoparticles, viral derived……
High Content Tier 1 Endpoints (Assessed between 24 and 120 hpf)

Morphological Malformations
  i.e. pericardial edema, yolk sac edema, body axis fin malformations, eye diameter

Circulation
Heart beat (rate)
Developmental progression
Embryo viability

Behavioral
spontaneous movement (18-24 hpf) onset and frequency
touch response (27 hpf)
motility
Automation: To Increase Throughput

Our Recent Technical Advances

• Embryo Production
• Embryo handling
• Microinjections
• Plate reader based assays
• Behavioral assays
C₆₀ Exposures and Response

Carbon Fullerenes:

- Mortality
- Pericardial Edema
- Yolk Sac Edema
- Fin Malformation

![Graph showing C₆₀ Concentration and response](image)

![Images of zebrafish control and exposed to C₆₀](images)
Light Exposure Increases $C_{60}$ Toxicity

![Graph showing % Mortality vs. $C_{60}$ Concentration (ppb)]

- Black line: $C_{60}$
- Gray line: $C_{60}$ dark

![Graph showing % Pericardial Edema vs. $C_{60}$ Concentration (ppb)]

- Black line: $C_{60}$
- Gray line: $C_{60}$ dark

* denotes statistical significance.
Oxidative Stress Response (Tier 2)

C60

Oxidative Stress?

Antioxidants Depletion (i.e. GSH)

Gene Expression Changes

Cell Death

Protein Damage/Dysfunction

Lipid peroxidation
GSH Precursor - NAC Offers Partial Protection
The Antioxidant Ascorbic Acid Offers Partial Protection

![Graphs showing cumulative % mortality and cumulative % pericardial edema against C₆₀ concentration (ppb)]
Chemical Depletion of Glutathione

Embryos Are More Sensitive to \( C_{60} \)
Oxidative Environment Impacts In vivo Cellular Death Response
C$_{60}$ Dose Determination

• Goal: to develop a method for detecting and quantifying C$_{60}$ associated with biological and aqueous samples.

• Analytical quantification of C$_{60}$ using LC-MS (Collaboration with Dr. Carl Isaacson and Dr. Jennifer Field – OSU EMT)

• Pooled 100 embryos per replicate

• Use of $^{13}$C-labeled C$_{60}$ surrogate to calculate losses during extraction method.
Water Concentration Declines Over Time

C₆₀ Embryo Water

Concentration (ppb)

Hours of Exposure
The $C_{60}$ LD$_{50}$ in embryonic zebrafish is 0.1 ng/mg.
General approach

• We focus on EC\textsubscript{100} (i.e. 100 % cardiovascular effect @ 5 days)

• Focus on early responses…when the endpoint is not visible

Example

• Zebrafish oligo arrays used to evaluate gene expression changes following exposure

• 200 ppb C\textsubscript{60} and 1% DMSO controls

• Expression evaluated at 12 & 24 hrs post exposure
Conclusions

• Cannot (yet) predict biological responses
• Many advantage by evaluating interactions/responses in vivo
  - multiple levels of organization
• Zebrafish: a discovery platform to define nanomaterial/biological Interactions from diverse sources
• Opportunities to define structure response relationships
• Extremely well-suited for whole animal mechanistic studies.
Current Nano Needs

- Efficient dissemination of shared materials
- Reduce the randomness of assessments
- Data sharing infrastructure
- Comparative analysis with shared data
- Define mode of actions of responsive NPs
- Develop predictive behavior models
- Test predictive models
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