# About OMICS Group

OMICS Group International is an amalgamation of Open Access publications and worldwide international science conferences and events. Established in the year 2007 with the sole aim of making the information on Sciences and technology 'Open Access', OMICS Group publishes 400 online open access scholarly journals in all aspects of Science, Engineering, Management and Technology journals. OMICS Group has been instrumental in taking the knowledge on Science & technology to the doorsteps of ordinary men and women. Research Scholars, Students, Libraries, Educational Institutions, Research centers and the industry are main stakeholders that benefitted greatly from this knowledge dissemination. OMICS Group also organizes 300 International conferences annually across the globe, where knowledge transfer takes place through debates, round table discussions, poster presentations, workshops, symposia and exhibitions.

# About OMICS Group Conferences

OMICS Group International is a pioneer and leading science event organizer, which publishes around 400 open access journals and conducts over 300 Medical, Clinical, Engineering, Life Sciences, Pharma scientific conferences all over the globe annually with the support of more than 1000 scientific associations and 30,000 editorial board members and 3.5 million followers to its credit.

OMICS Group has organized 500 conferences, workshops and national symposiums across the major cities including San Francisco, Las Vegas, San Antonio, Omaha, Orlando, Raleigh, Santa Clara, Chicago, Philadelphia, Baltimore, United Kingdom, Valencia, Dubai, Beijing, Hyderabad, Bengaluru and Mumbai.

# Toxicity Evaluation of Carbon Nanotubes in J774 Mouse Macrophages Utilizing a Proteomic Approach

Dharani Das

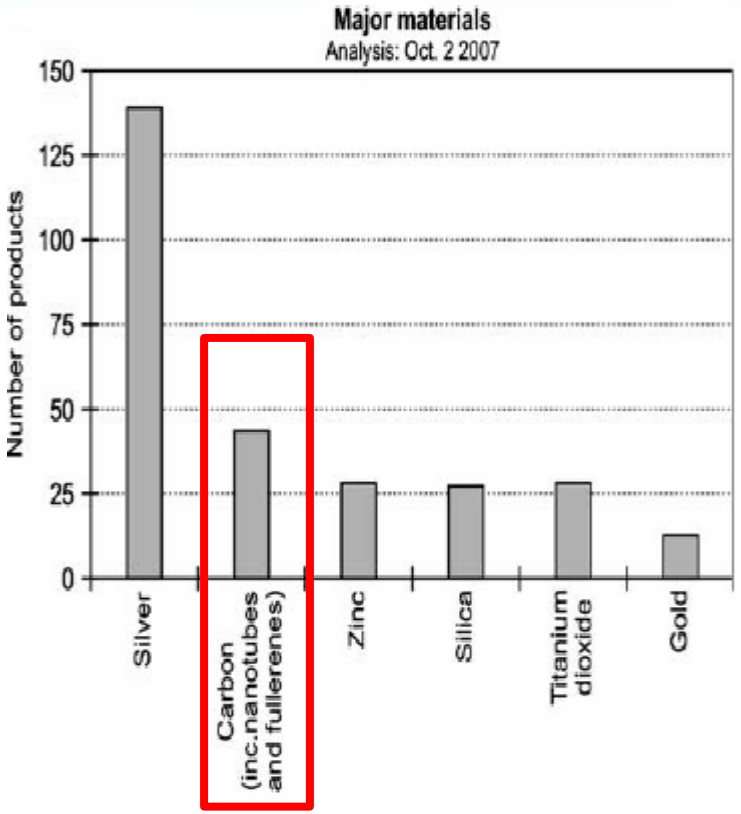
***Analytical Biochemistry and Proteomics Laboratory, Mechanistic Studies Division, Environmental Health Science and Research Bureau, HECSB, Health Canada***



Date: 6th August 2014

Canada

# Why CNTs?




*From Hansen et al., Ecotoxicology, (2008) 17(5):438-47 2008*

Number of consumer products with CNTs:

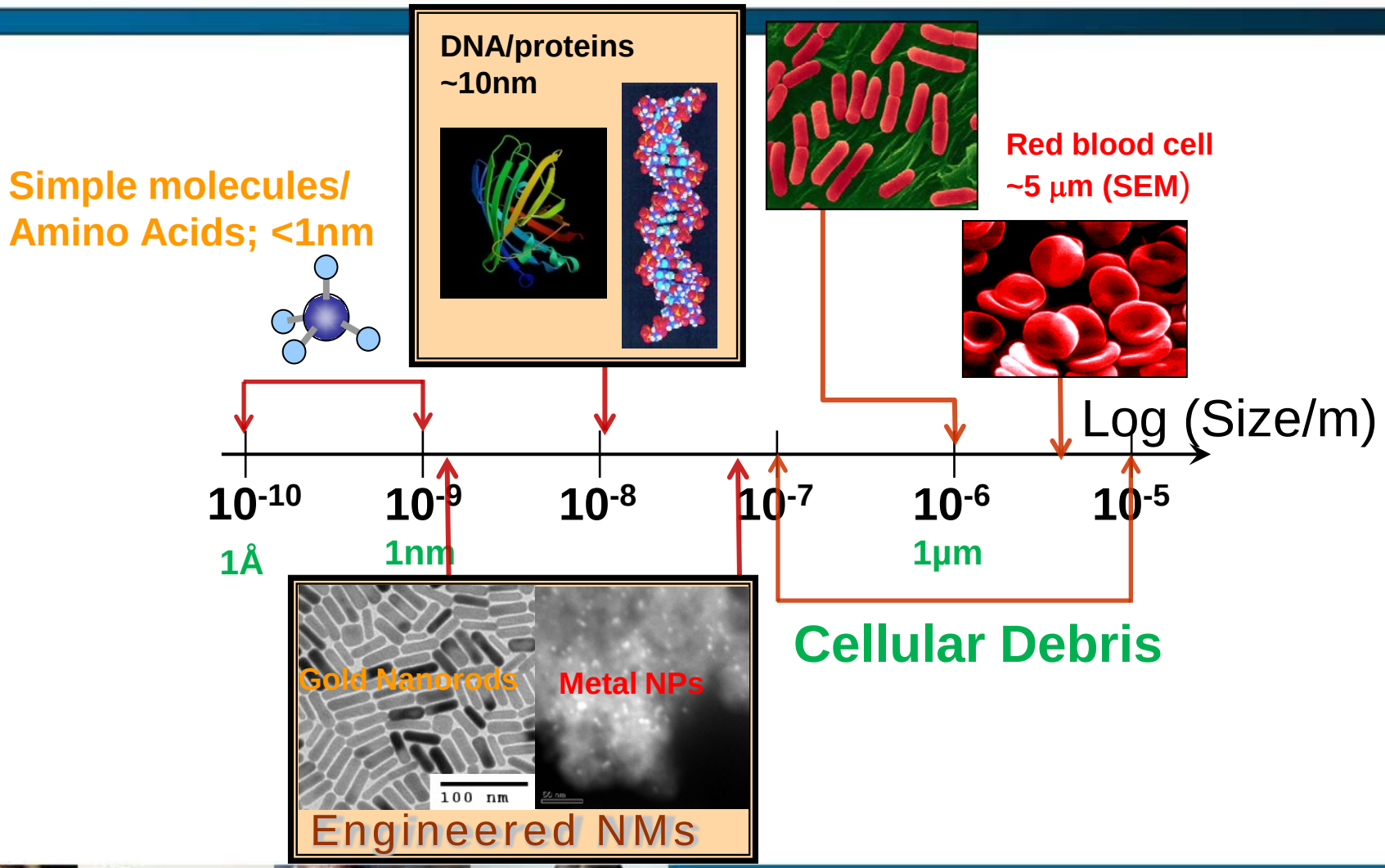
580 consumer products

4Therapeutic products (FDA Approved)

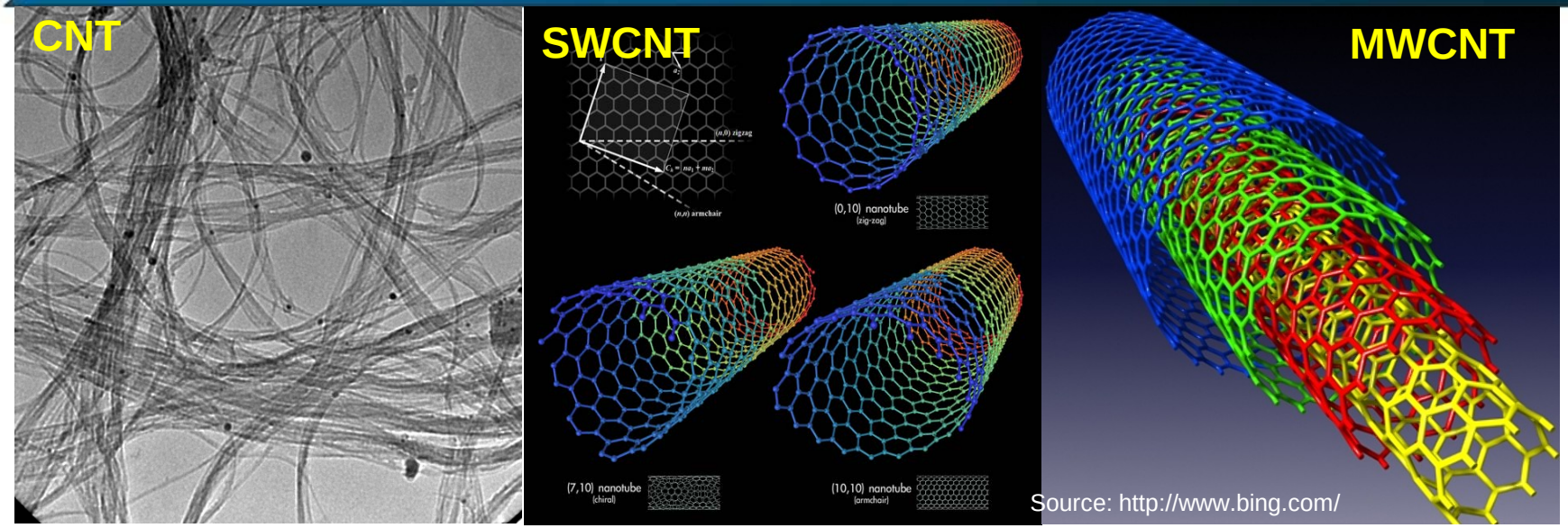
## Health Risks of CNTs

- Increased use of CNTs into consumer products
- Potential hazards to humans during product **lifecycle**

# Introduction

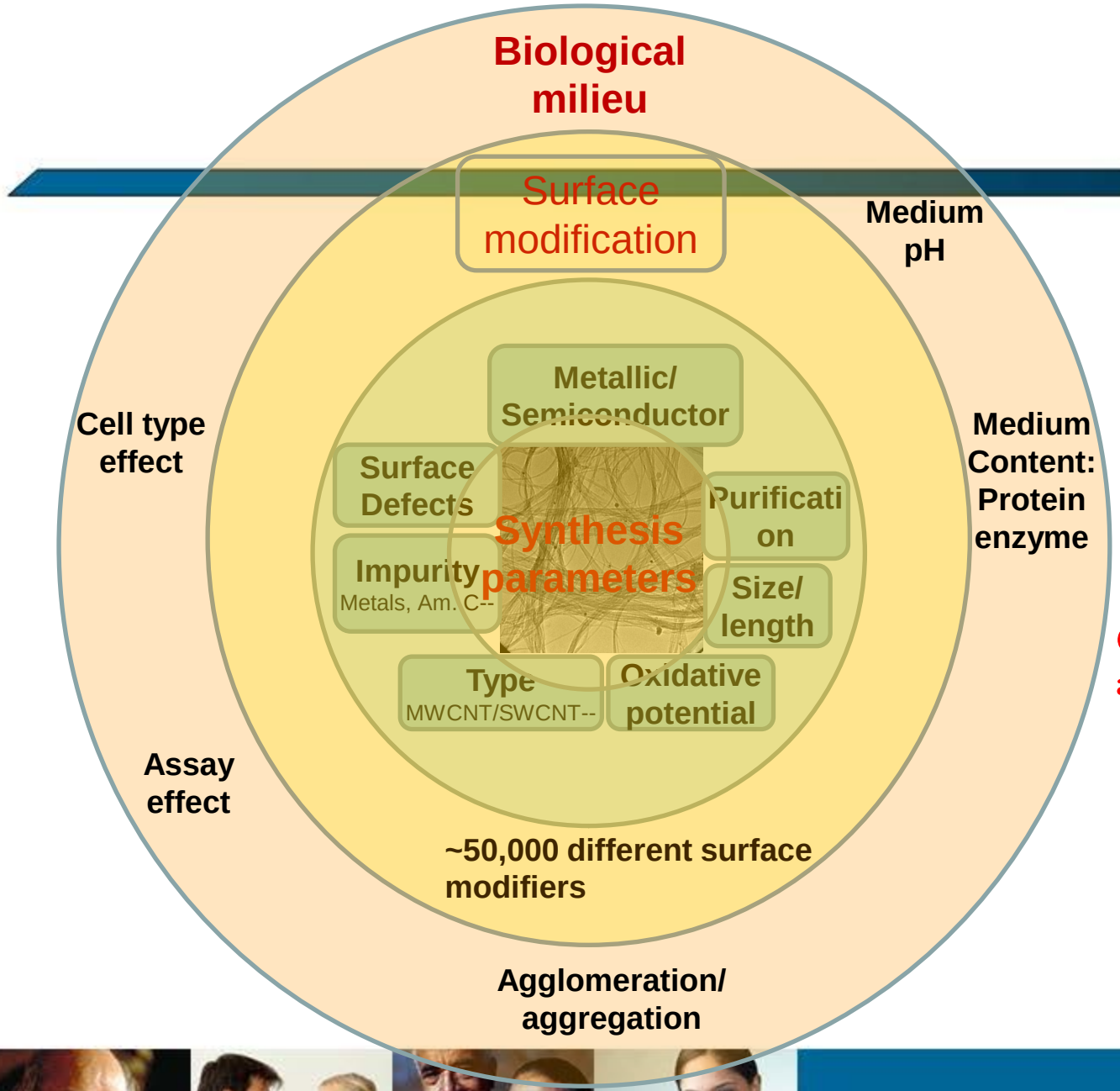



# CNT Structures




- **Long fibre like structures with diameters <10 nm and µm in length**
- **Natural (in DEPs, natural gas combustions) and Engineered**
- **Synthesized mainly in two forms: single walled (SWCNT) and multi-walled (MWCNT)**

- Unique nano-specific properties Varies with:
  - Size
  - Shape
  - Surface functionality




Care must be taken in analyzing and interpreting in vitro results

(Breznan et al., *Tox. Invitro*, under review; Kumarathasan et al., *Nanotoxicol.* 2014)

# Does exposure to CNTs pose a threat to human health?

- Exposure to CNTs causes inflammatory and fibrotic reactions (Literature Overview)
- Hazardous fibre characteristics: High aspect ratio (long, thin and biopersistannt)
  - May generate asbestos-like effect
- The concern about adverse human health effects upon exposure is REAL!!!

# Knowledge gap

- The CNTs toxicity results are sometimes contradictory

(For example, some *in vitro* experiments showed cytotoxicity of CNTs when exposed to lung epithelial cells, keratinocytes and macrophages *Monteiro-Reviere et al., 2005, Kagan et al., 2006, Sarkar et al., 2007; Hirano et al., 2008; Jacobsen et al., 2008; Herzog et al., 2009; Yang et al., 2009*. On contrary, some results show no toxicological responses upon exposure to cells or test animals *Flahaut et al., 2006; De Nicola et al., 2007; Pulskamp et al., 2007; Yang et al., 2008*).

- Often inadequate characterization data; thus comparison between studies are impossible

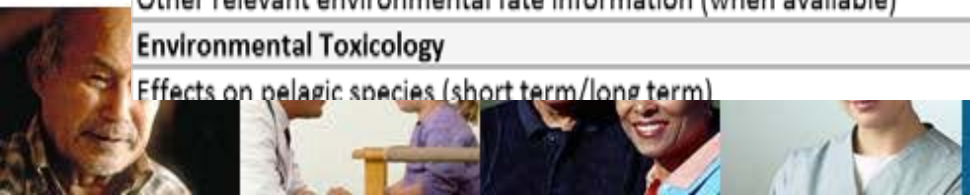
- In vitro toxicity is cell type and assay dependent
  - Cellular toxicity results does not reveal enough information on toxicological pathways

- In vivo studies are expenssive and can not be used to test so many varieties of (same)materials

- Often In vitro results are not consistent with in vivo finding; thus *in vitro* to *in vivo* extrapolation is impossible at this stage

| OECD endpoints according to the Guidance Manual for Sponsors (GMS) | GMS- data requirements |
|---|---|
| NM name | must be completed |
| CAS No | if available |
| structural formula/molecular structure | must be provided |
| composition of NM being tested including purity, known impurities or additives | must be provided |
| basic morphology | must be provided |
| description of surface chemistry | if feasible |
| major commercial uses | as completely as possible |
| known catalytic activity | should be described |
| method of production | must be described |
| **Identification, source, logistics of distribution (Guidance manual for Sponsors §41 and 42)** | |
| known aspects: manufacturer, facility location, lot number, other, see above | must be completed |
| records on distribution, shipment, storage | must be completed |
| quality of material: homogeneity within bottle/ between bottles | must be completed |
| quality of material: stability, short-term and long-term | must be completed |
| quality of material: stability, monitoring | must be completed |
| **Physical-chemical Properties and Material Characterization** | |
| Agglomeration/aggregation | must be addressed |
| Water Solubility/Dispersibility | must be completed |
| Crystalline phase | must be completed |
| Dustiness | must be addressed |
| Crystallite size | must be addressed |

| | |
|---|---|
| Representative Electron Microscopy (TEM) picture(s) | must be addressed |
| Particle size distribution – dry and in relevant media | must be completed |
| Specific surface area | must be completed |
| Zeta potential (surface charge) | must be completed |
| Surface chemistry | must be completed |
| Photocatalytic activity | must be addressed |
| Pour density | must be addressed |
| Porosity | must be addressed |
| Octanol-water partition coefficient | must be addressed |
| Redox potential | must be addressed |
| Radical formation potential | must be addressed |
| Other relevant Physical-Chemical Properties and Material Characterization information (where available) | must be addressed |
| **Environmental Fate** | |
| Dispersion stability in water | must be addressed |
| Biotic degradability | must be addressed |
| Identification of degradation product(s) | must be addressed |
| Further testing of degradation product(s) as required | must be addressed |
| Abiotic Degradability and Fate | must be addressed |
| Adsorption-Desorption | must be addressed |
| Adsorption to soil or sediment | must be addressed |
| Bioaccumulation potential | must be addressed |
| Other relevant environmental fate information (when available) | must be addressed |
| **Environmental Toxicology** | |
| Effects on pelagic species (short term/long term) | |



# Knowledge gaps for risk assessment of CNTs

Occupational and environmental exposure-related effects

## Hazard X Exposure = Risk

- Environmental and workplace exposure data are required, and more precise measurement methods are needed. Such exposure data would guide long-term animal studies to determine the time course and dose response for possible development of fibrosis, lung cancer, or mesothelioma
- Some NPs are sometime non-cytotoxic even when the cells are loaded with them (TEM)
- Elucidation of mechanisms of action through biomarkers analysis would be useful in surveillance

- Identification of relationships between physico-chemical properties and bioactivity would assist in progress toward "Safety by Design"

Proteomic analysis is one such approach that has a great potential to reveal mechanistic pathways of CNT toxicity and can also identify better biological descriptors of potencies

# Objectives

- Study the protein profile changes in lysates of J774 cells pertaining to exposure to CNT variants (unmodified and surface functionalized)

- Identify the candidate biomarker(s) and possible mechanistic pathway of toxicity

- Study the association between physico-chemical and surface properties on toxicity

# Materials and Techniques

## CNTs

Pristine SWCNT (NRC, Ottawa)
Pristine MWCNT (Sun Nanotech)
Oxidized SWCNT & MWCNT ($H_2SO_4/HNO_3$)

## Cell line

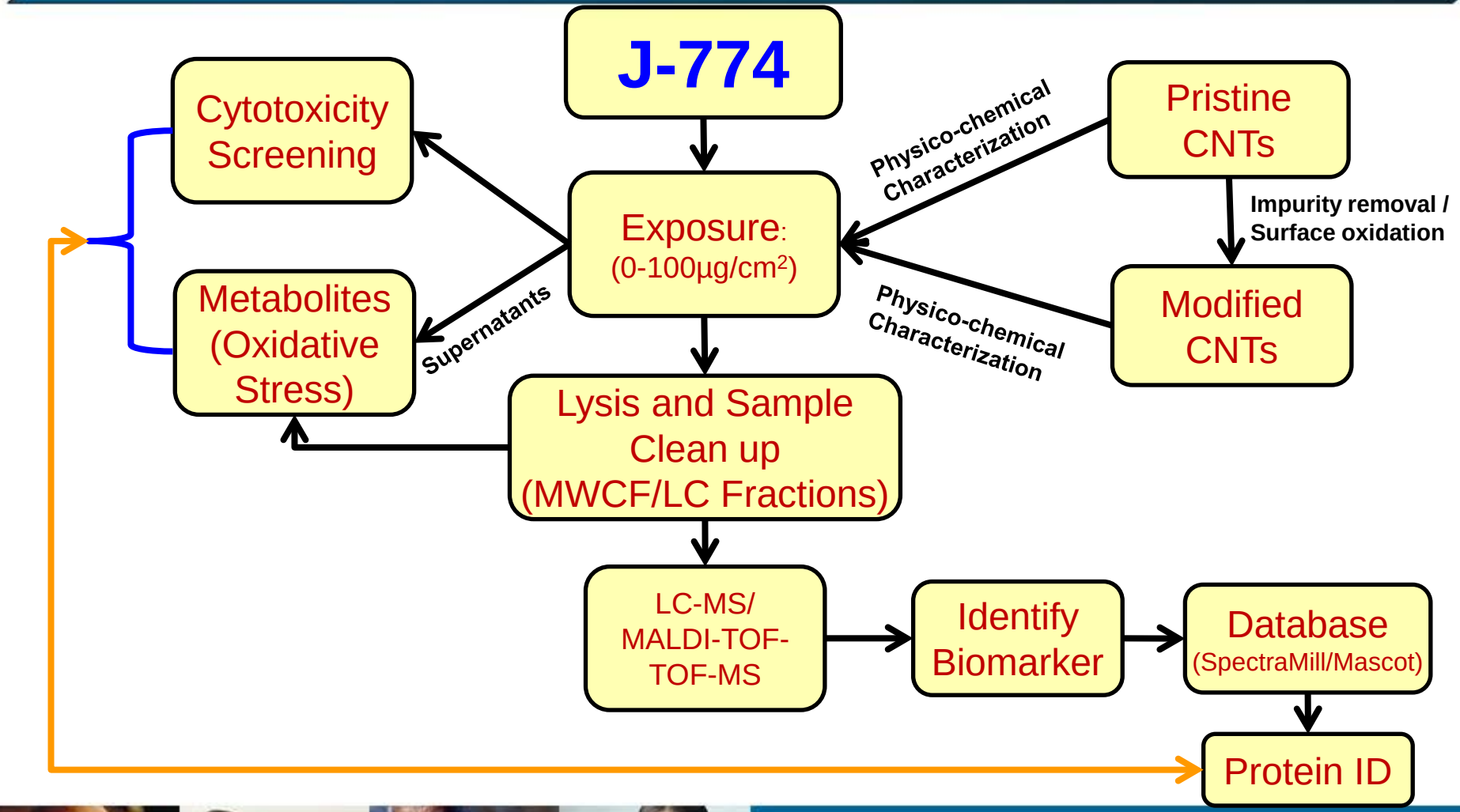
J774, murine macrophages

**4 Cytotoxicity Assays**

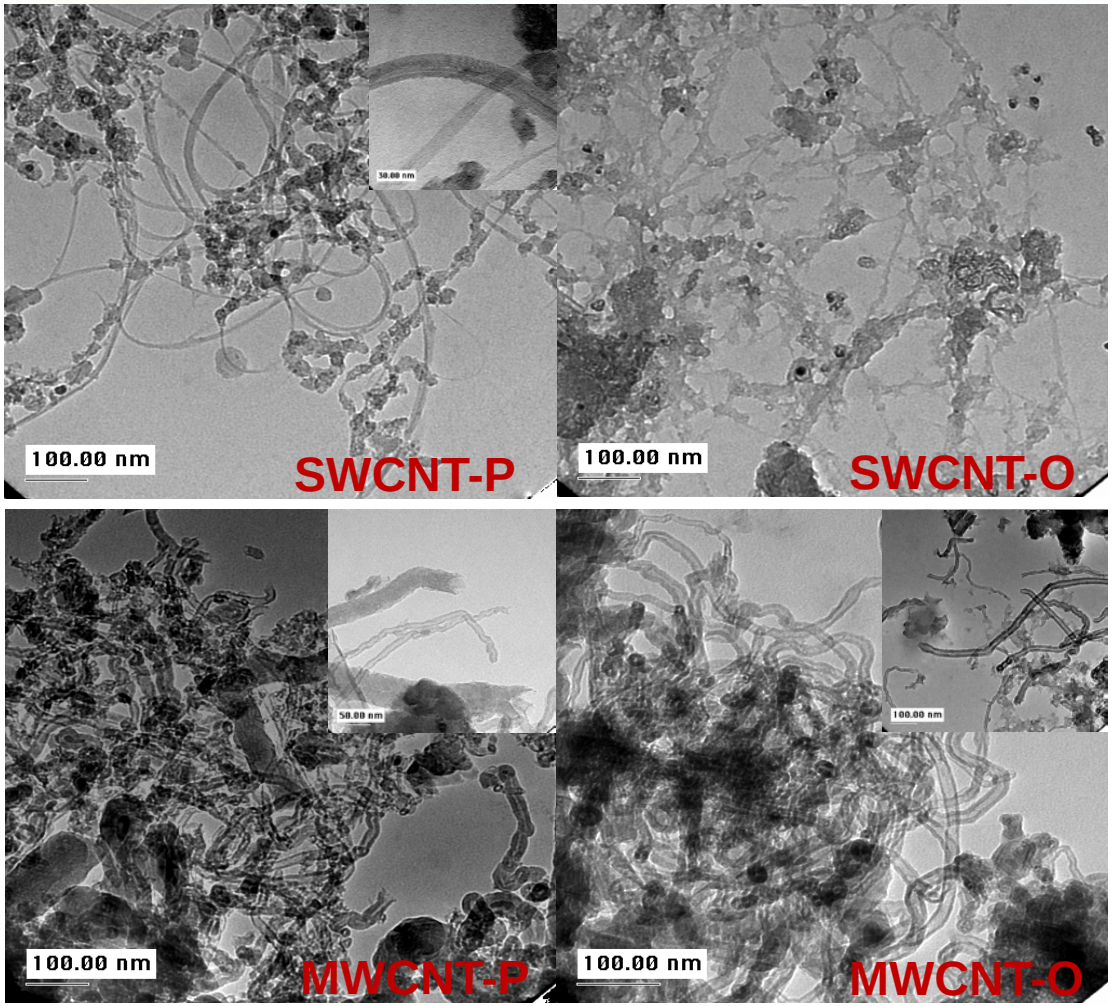
## Biomarker Analysis

Shot-gun proteomics (2D-LC-MS; MALDI-TOF-TOF-MS)
Clinpro Tools
Oxidative stress metabolites (HPLC-CoulArray)

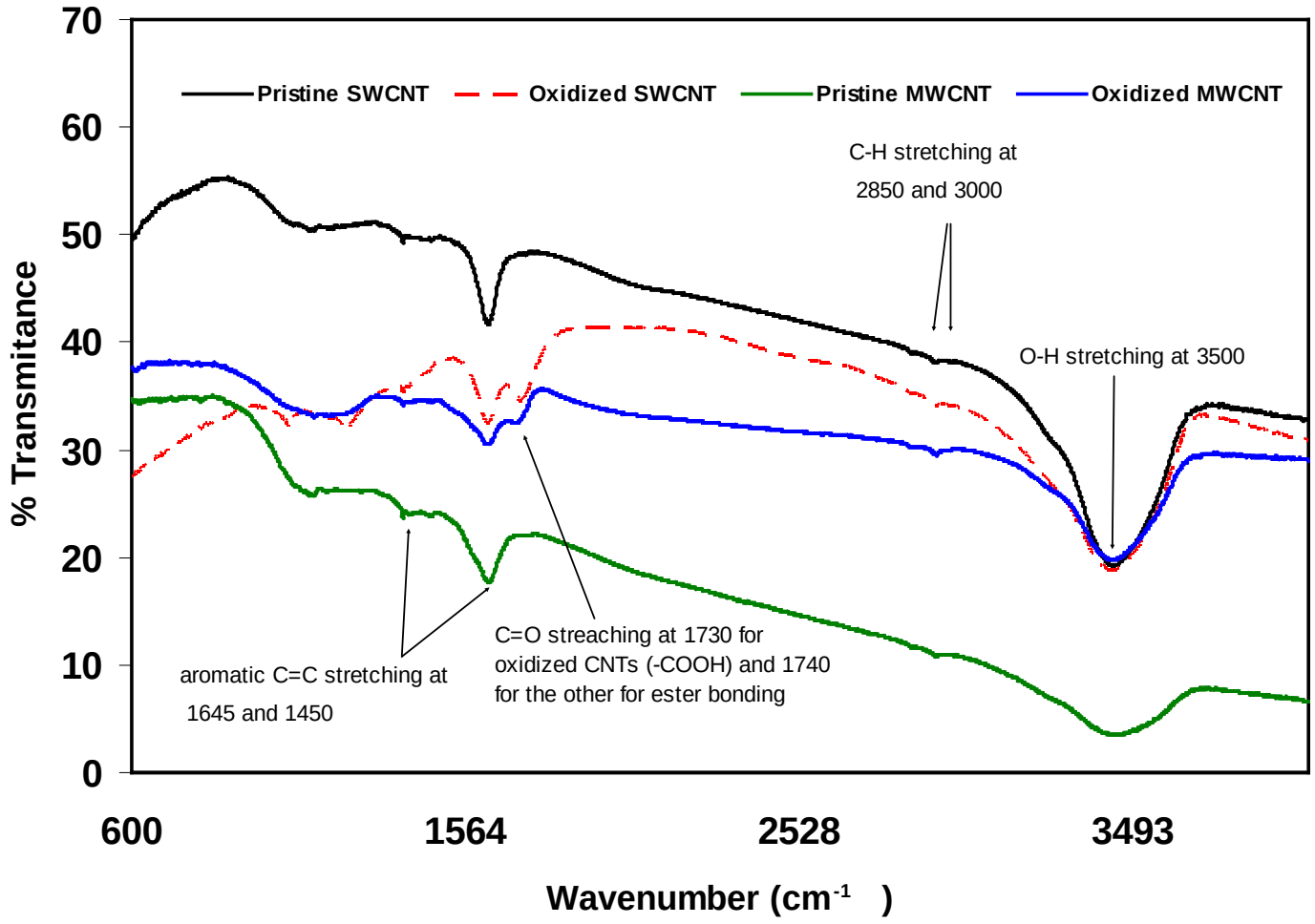
# Workflow


J-774
Cytotoxicity Screening
Metabolites (Oxidative Stress)
Supernatants
Exposure: (0-100µg/cm2)
Physico-chemical Characterization
Pristine CNTs
Impurity removal / Surface oxidation
Modified CNTs
Physico-chemical Characterization
Lysis and Sample Clean up (MWCF/LC Fractions)
LC-MS/ MALDI-TOF-TOF-MS
Identify Biomarker
Database (SpectraMill/Mascot)
Protein ID

# Morphology before/after surface oxidation




# Fourier Transformed Infrared Spectroscopy

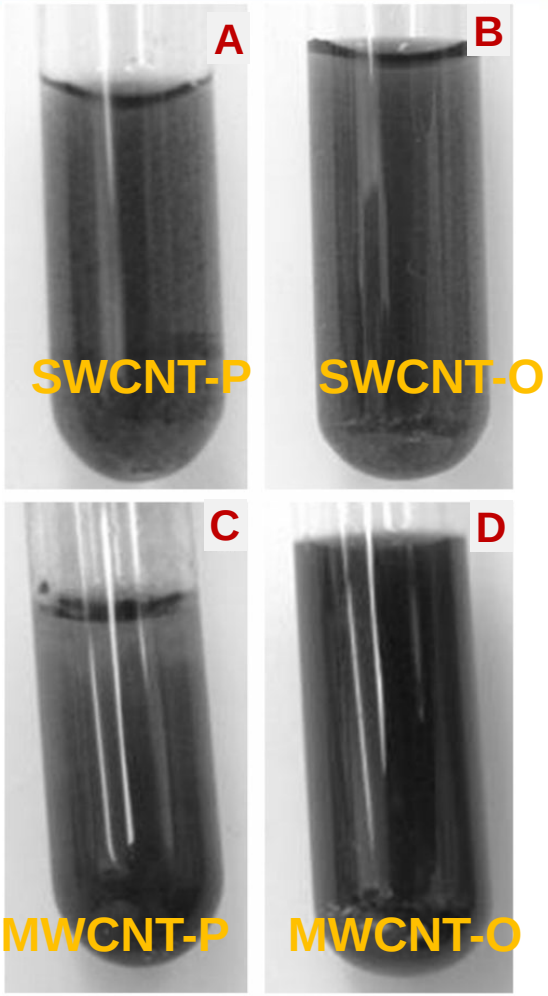



# Surface properties of CNTs

| Sample | Surface Area, $m^2/g$ | %Co | %Fe | %Ni | %Mo |
|---|---|---|---|---|---|
| **SWCNT-P** | **89** | 1.24 | 0.32 | 1.16 | 0.28 |
| **SWCNT-O** | **21** | 0.21 | 0.1 | 0.188 | 0.04 |
| **MWCNT-P** | **106** | BDL | 1.08 | 0.62 | BDL |
| **MWCNT-O** | **23** | BDL | 0.52 | 0.44 | BDL |

- Surface area and metal impurities decrease with oxidation

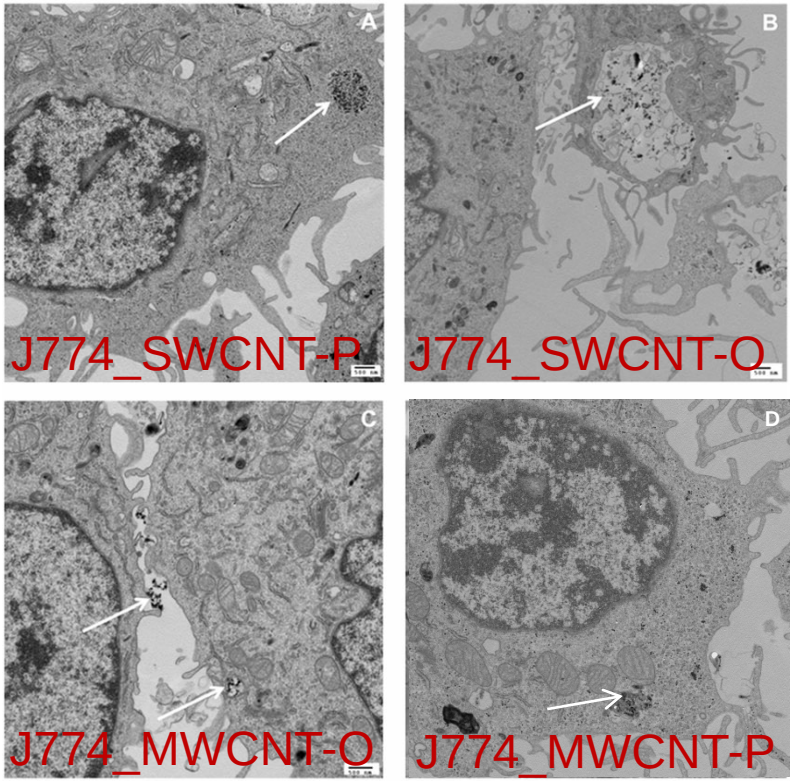
# Dispersion of CNTs in particle buffer (25 µg/mL Tween-80, 0.19% NaCl in water)




## DLS measurement of CNTs suspended in DMEM + 5% FBS

| CNTs [0.05 mg/ml] | Diameter (nm) | PDI |
|---|---|---|
| **SWCNT-P** | 7778 | 1 |
| **SWCNT-O** | 1511 | 1 |
| **MWCNT-P** | 2918 | 0.85 |
| **MWCNT-O** | 188.3 | 0.35 |

# Intracellular localization of CNTs

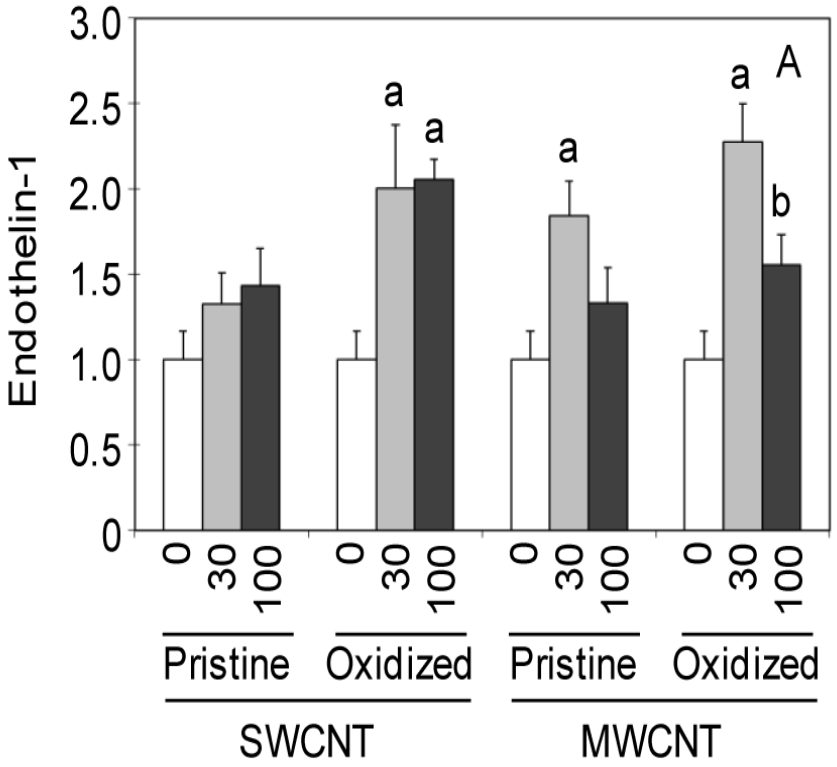



# Candidate biomarker expressions with CNT exposures

Highly potent vasoconstrictor

| Proteins | Expressions | Cellular Functions |
|---|---|---|
| Endothelin-1 | ⬆ | Inflammatory and mitogenic peptide |
| Lactate dehydrogenase | ⬇ | Metabolic process / membrane permeability |
| Immunoglobulin kappa chain | ⬆ | Antigen binding and effector function |
| Immunoglobulin heavy chain | ⬆ | Antigen binding and effector function |
| T-cell receptor *β*-chain | ⬆ | Antigen presentation |
| MHC II bound peptide fragment | ⬆ | Antigen presentation |
| *α*-Endosulfine | ⬆ | Protein phosphatase inhibitor, interferes with mitosis |
| *β*-Actin | ⬇ | Cytoskeletal rearrangement |
| Tropomodulin | ⬇ | Cytoskeletal rearrangement |
| T-cell receptor *α* V region | ⬇ | Antigen presentation |
| T-cell receptor delta chain | ⬇ | Antigen presentation |
| Homeobox protein 4.2 | ⬇ | Transcription factor, cell survival, proliferation |
| 65-kDa macrophage protein | ⬆ | - |
| Gamma actin | ⬆ | Cytoskeletal rearrangement, adhesion, intracellular trafficking and signal transduction |
| Brain abundant, membrane attached signal protein 1 | ⬆ | - |
| Prothymosin alpha | ⬆ | Cell proliferation and immune regulation |
| Heat shock 70kDa protein 8 isoform 1 variant | ⬆ | Heat shock response is highly conserved defence mechanism of cells against conditions of environmental stress, such as heat and oxidative shock |
| YWHAZ protein | ⬇ | |
| Glyceraldehyde-3-phosphate dehydrogenase | ⬇ | Glycolytic pathway and energy production, Other functions such as membrane fusion, microtubule bundling, phosphotransferase activity, nucleic acids binding, etc. |

# CNT exposure increased endothelin-1 Expression



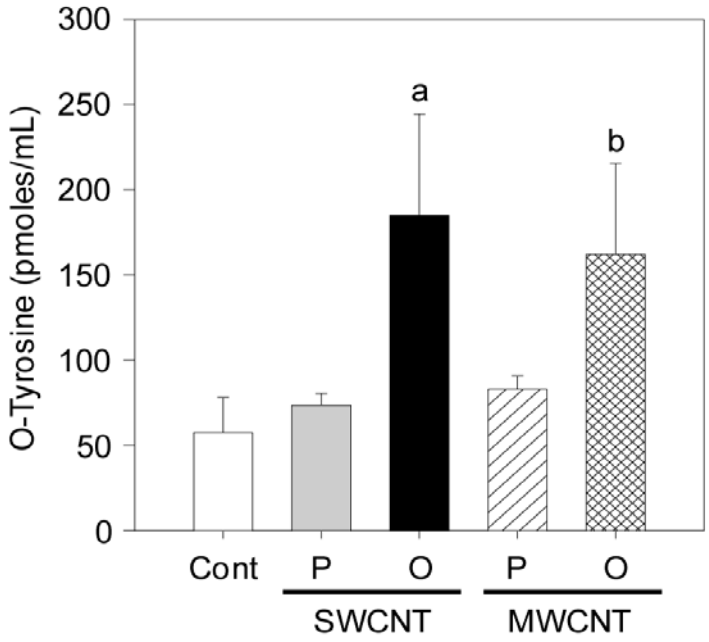

**Mass spectral peak area profiles of endothelin-1**

# Differentially Expressed Proteins

| SWCNT-P | SWCNT-O | MWCNT-P | MWCNT-O |
|---|---|---|---|
| | mCG17543, isoform CRA_a | | |
| | CAP, adenylate cyclase-associated protein 1, isoform CRA_b | | |
| Enolase 1 variant | Enolase 1 variant | Enolase 1 variant | Enolase 1 variant |
| M2-type pyruvate kinase | M2-type pyruvate kinase | M2-type pyruvate kinase | M2-type pyruvate kinase |
| Rho, GDP dissociation inhibitor (GDI) beta, Isoform CRA-a | Rho, GDP dissociation inhibitor (GDI) beta, isoform CRA_a | Rho, GDP dissociation inhibitor (GDI) beta, isoform CRA_a | Rho GDP dissociation inhibitor (GDI) beta, isoform CRA_a |
| TMSB4X protein | mCG22383, isoform CRA_e | mCG22383, isoform CRA_e | mCG22383, isoform CRA_e |
| | Cofilin 1, non-muscle | Cofilin 1, non-muscle | Profilin 1, isoform CRA_b |
| Heat shock protein HSP 90-beta | Heat shock protein HSP 90-beta | Heat shock protein HSP 90-beta | |
| | Heat shock protein 1-b | | Heat shock protein 1-b |
| | Heat shock protein 8 | | Heat shock protein 8 |
| | Filamin, alpha | Filamin, alpha | actr2 protein |
| | | | Brain creatine kinase |
| | | | mCG134299,isoform CRA_a |
| | | | mCG17007 |
| | | | Phosphoglyserate kinase 1 |
| | | | Tyrosine 3-monooxygenase, isoform CRA_d |

# Analysis of the reactive oxygen species marker o-tyrosine

- Engineered NMs perturb the oxidative balance of cells, resulting in very large concentrations of intracellular reactive oxygen species (**ROS**) and reactive nitrogen species (**RNS**) (Marquis et a., 2009, Kumarathasan and Das et al., 2012); Or, **RONS?**
- **ROS** can cause damage to cellular macromolecules (proteins, lipids or nucleic acids), resulting in abnormal cellular function.




**Oxidized > Pristine**

# Potencies of Carbon Nanotubes

| CELL | PM | β | | LDH/ATP | β | | CTB/BrdU |
|---|---|---|---|---|---|---|---|
| | | LDH | ATP | | CTB | BrdU | |
| J774 | SW-P | -0.054 | -0.119 | **-0.087** | -0.017 | -0.104 | -0.061 |
| | SW-O | -0.017 | -0.053 | -0.035 | -0.042 | -0.124 | **-0.083** |
| | MW-P | -0.053 | -0.084 | **-0.069** | -0.028 | -0.047 | -0.038 |
| | MW-O | -0.017 | -0.066 | -0.042 | -0.060 | -0.117 | **-0.089** |
| | $TiO_2$ | -0.005 | -0.015 | -0.010 | -0.004 | -0.013 | -0.009 |
| | $SiO_2$ | -0.018 | -0.028 | -0.023 | -0.019 | -0.037 | -0.028 |

**Fold-effect = (Dose + 1)$^{\beta}$; Kumarathasan et al, Nanotoxicology, 2014**

- Distinct results were observed with multiple cell line and multiple endpoints; which highlight the difficulty in drawing generalized conclusions

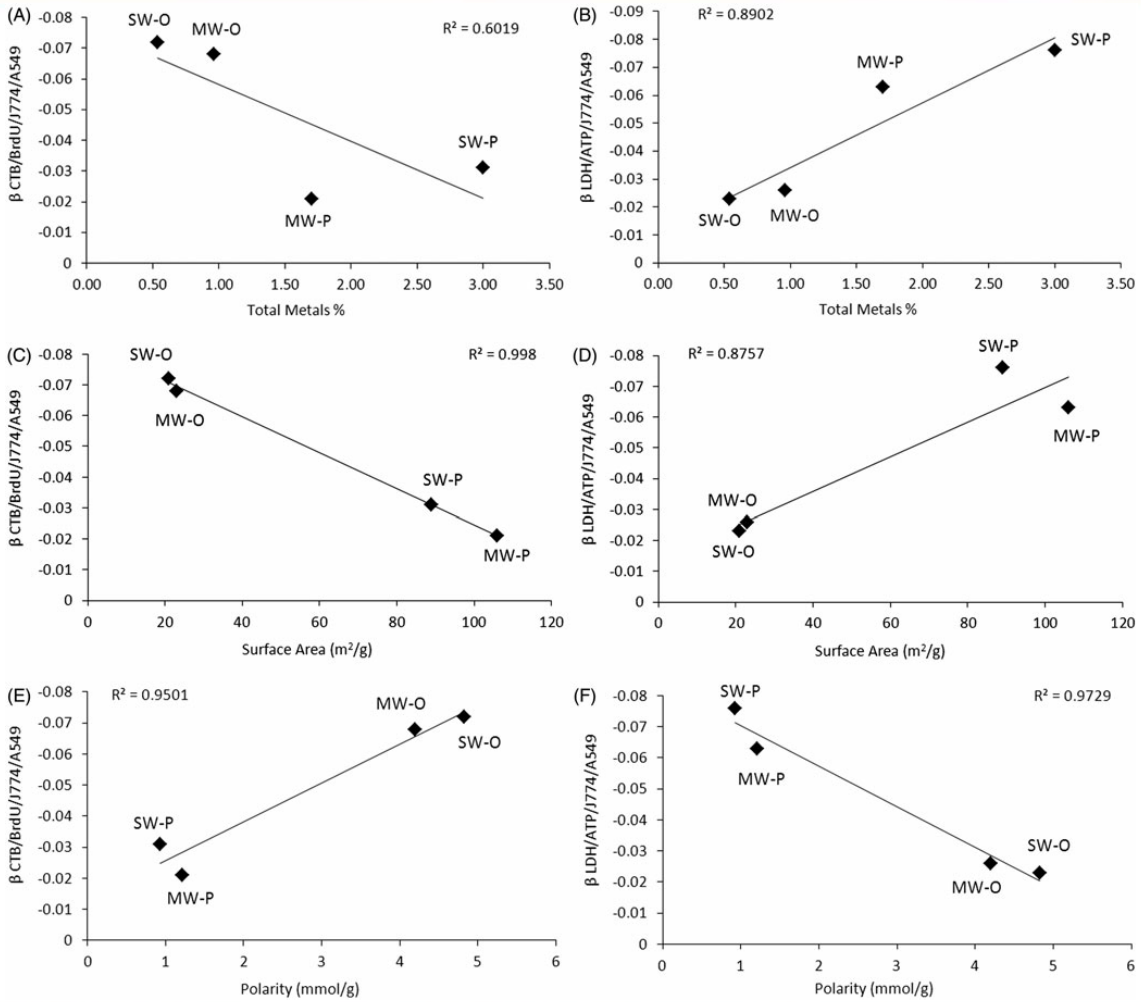
**Possible solution:**

multiple cell lines and multiple end point

Relative potency ranking (2 cell lines and 4 assays)

**SWCNT-O > MWCNT-O > SWCNT-P > MWCNT-P**

# Correlation of cytotoxic potency of CNT variants with physico–chemical characteristics.




Kumarathasan et al, *Nanotoxicology*, 2014

# Conclusions

- More proteins were expressed in oxidized CNTs than their pristine counterparts
- Expression of several Heat Shock Proteins in CNT-exposed cells indicates oxidative stress under the exposure conditions
- Higher levels of *o*-Tyrosine in oxidized CNTs than their pristine counterparts indicate oxidative stress as one of the pathways of CNT toxicity
  - supports the above hypothesis
- Relative potency ranking from cytotoxicity assays follows the order: SWCNT-O > MWCNT-O > SWCNT-P > MWCNT-P
  - Higher potency of oxidized CNTs was attributed to surface polarity
- The proteomic/metabolomic data support the cytotoxicity results obtained using multiple cell lines and assays

# Acknowledgements

**Analytical Biochemistry & Proteomics Laboratory, Health Canada**

**Prem Kumarathasan**

**Inhalation Toxicology Laboratory, Health Canada**

Renaud Vincent

Dalibor Breznan

*Nothing gets done without collaboration!!!!!*

**Steacie Institute of Molecular Sciences, NRC**

Benoit Simard

Jingwen Guan

**University of Ottawa**

Nimal De Silva

Peter Ripstein

**CMP Nano funding, Health Canada**

# THANKS!!!

# Questions?

# Let Us Meet Again

We welcome you all to our future conferences of OMICS Group International

Please Visit:

www.omicsgroup.com

www.conferenceseries.com

www.proteomicsconference.com