Cell-Based Toxicity Assay-on-Chip for the Next-Generation CMOS Technology

(Task Number: 425.037)

PIs:

- **Shyam Aravamudhan, Nanoengineering, NC A&T**
- **Adam R. Hall, Nanoscience, UNC/Greensboro**
- **Shanthi Iyer, Nanoengineering, NC A&T**
- **Marinella Sandros, Nanoscience, UNC/Greensboro**
- **Ethan W. Taylor, Nanoscience, UNC/Greensboro**

Graduate Students:

- **Komal Garde, Nanoengineering, NC A&T**
- **Smith Woosley Nanoengineering, NC A&T**
- **Karshak Kosaraju, Nanoscience, UNC/Greensboro**

Other Researchers:

- **Steven Crawford, Research Technician, Nanoengineering, NC A&T**

Cost Share (other than core ERC funding):

- **25% cost-share from the Joint School of Nanoscience e and Nanoengineering, a collaboration between NCA&T and UNC/Greensboro**

Objectives

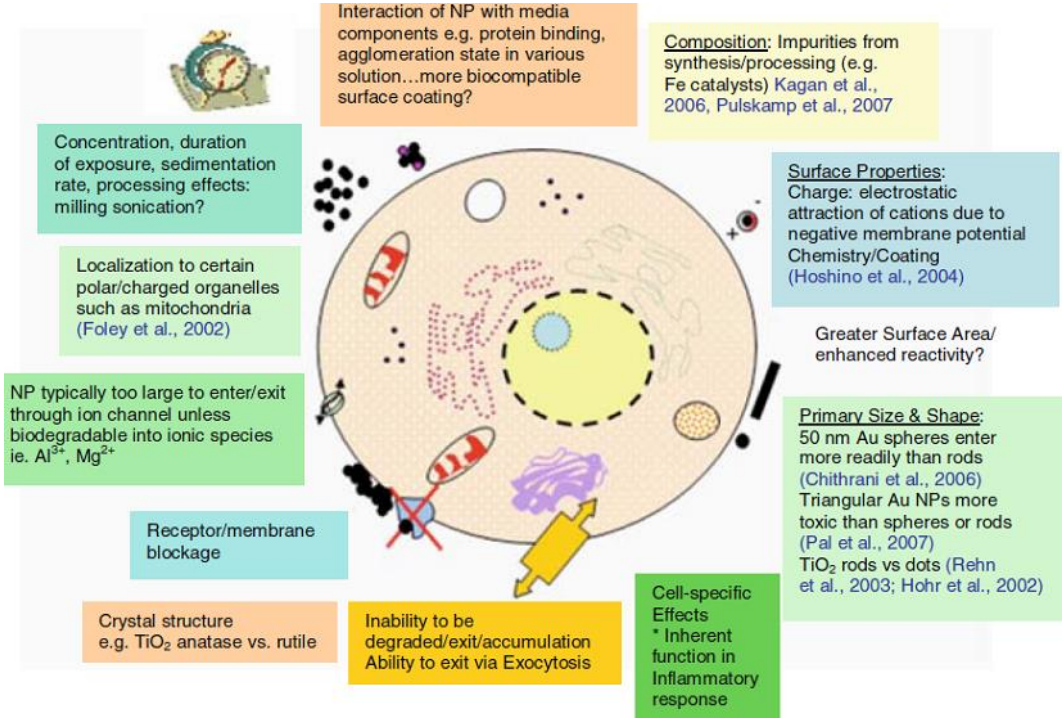
- **Long-term objective**
  - **Understand toxicity and cell uptake of engineered nanomaterials (ENs) used/to be used in the semiconductor industry**
  - **Cell assays and analytical/microscopic techniques to assess rapidly influence of physiochemical properties on ESH**
- **Year 1 focus**
  - **Short-term/acute (< 3 weeks) *in vitro* assessment of critical biological consequences (CBCs) using cytotoxic assays and cell uptake studies for varying physiochemical ENs**
- **Critical questions which need to be addressed…. (Schrurs et al. Nature Nanotechnology, 2012)**
  - **Do ENs penetrate into cells and how does intracellular trafficking of ENs occur?**
  - **Does cytotoxicity of ENs vary with cell type?**
  - **Is cytotoxicity driven by physiochemical factors such as size, aggregation, shape, composition, dose and time?**

ESH Metrics and Impact

1. **Reduction in the use or replacement of ESH-problematic materials (CNTs, CMP nanomaterials and other oxides)**
   - **Conducting comprehensive physicochemical characterization (of size, dose, aggregation, composition, crystallinity, surface properties) and toxicity assays to identity problematic materials (based on cell type)**
   - **We also identify and study subtle cellular variations, which may not result in cell death using cell uptake studies (dose - less than ppm levels)**
2. **Reduction in emission of ESH-problematic material to environment**
   - **Assessing starting nanomaterial's physiochemical properties including impurity content to remove unsafe by-products from the environment**
   - **Enable safer design and use of nanomaterials in work environments**
3. **Reduction in the use of natural resources (water and energy)**
4. **Reduction in the use of chemicals**

Understanding Nanotoxicity

- **Number of physicochemical properties and mechanisms can occur at the surface of nanomaterial and inside a cell, thus affecting toxicity**
- **Variability is huge due to different properties, cell types, processing and assay conditions**
- **Consortium approach with comprehensively characterized materials and same cells is ideal**
- ***In vitro* vs. *In vivo* assays**
- **Establish dose-response relationships and do predictive nanotoxicity**



Schematic representation of some possible interactions of nanomaterials with a cell in culture media (Schrand et al., 2009. Ch. in Safety of Nanoparticles, T.J. Webster (ed.))

Experimental Method and Approach

- **Nanomaterials investigated so far**
  - **Silica nanoparticles (Sigma No. 637246)**
  - **ZnO nanoparticles (Sigma No. 544906)**
  - **Au nanorods (Sigma, No. 716839)**
  - **Multi-Wall CNTs (Sigma 698849, 659258 and Consortium/UTD)**
  - **Silica and ZnO NPs in ultrapure water; Au nanorods used without any modification; MWCNTs with/without surfactants**
- **Cells studied**
  - **NIH/3T3 fibroblasts and PC-12 neurons from ATCC**
- **Physiochemical characterization**
  - **Size, shape, crystallinity, impurities and surface properties**
- **Cytotoxicity**
  - **Study cell membrane integrity and cell viability**
- **Microscopic and analytical techniques**
  - **Study whether nanomaterials are taken up by cells**

Experimental Results

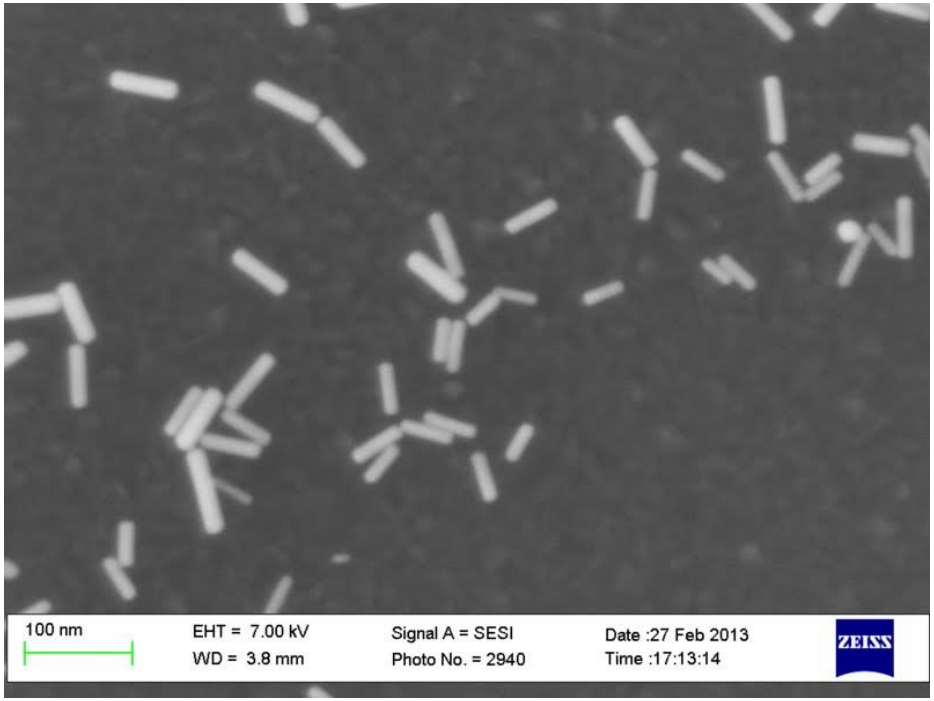
- **Physiochemical Characterization of starting materials**
  - **DLS (size, distribution, aggregation state), ICP-OES (impurities), XRD (crystallinity), VP-SEM and HRSEM (structure and morphology)**
- **Size, surface property and impurities - Important role in cytotoxicity as the surface area and aggregation state influence cytotoxic responses**

Physical properties of the different nanomaterials

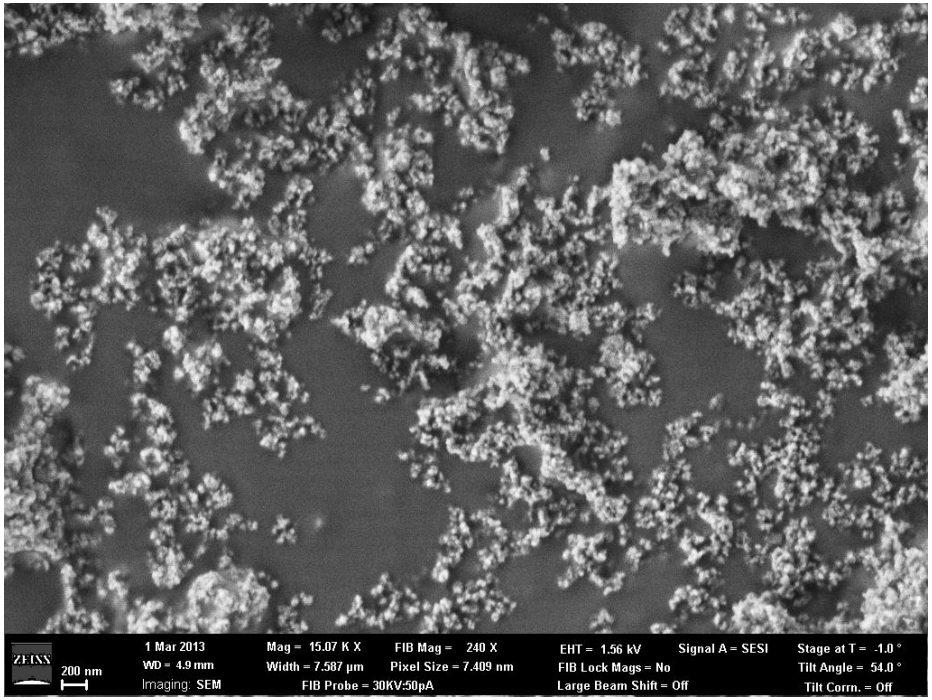
Material	Type	Size (nm)	Crystallinity	Surface
Silica	Particle	5-15	Amorphous	Pristine
ZnO	Particle	<100	Crystalline	Pristine
Au	Rod	10 by 45	Crystalline	CTAB
MWCNTs	Tube	6-13 by 2.5-20	Crystalline	Pristine
MWCNTs	Tube	100-170 by 5-9	Crystalline	Pristine

Characterization of Nanomaterials

- **Structural and morphological characterization of starting nanomaterials with and without modification**

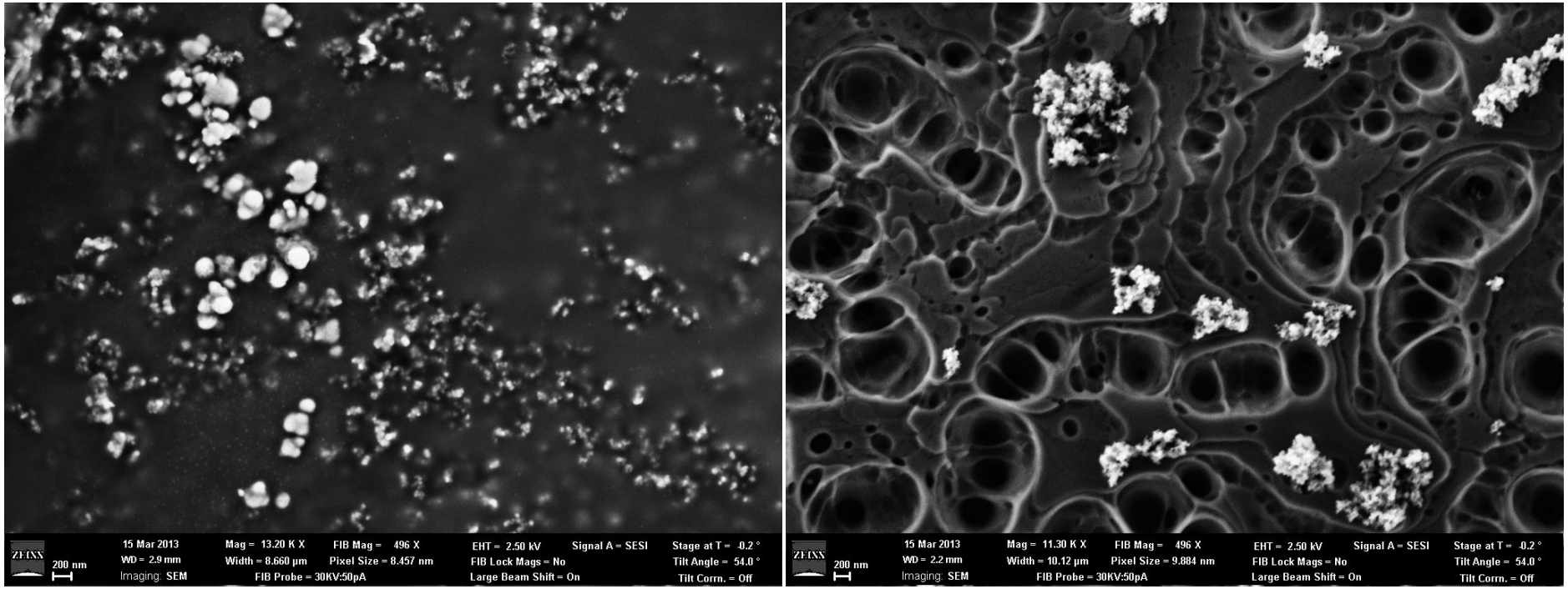


HRSEM image of as-obtained Au NRs in CTAB. Rods are about 10 nm in diameter and 45 nm in length.



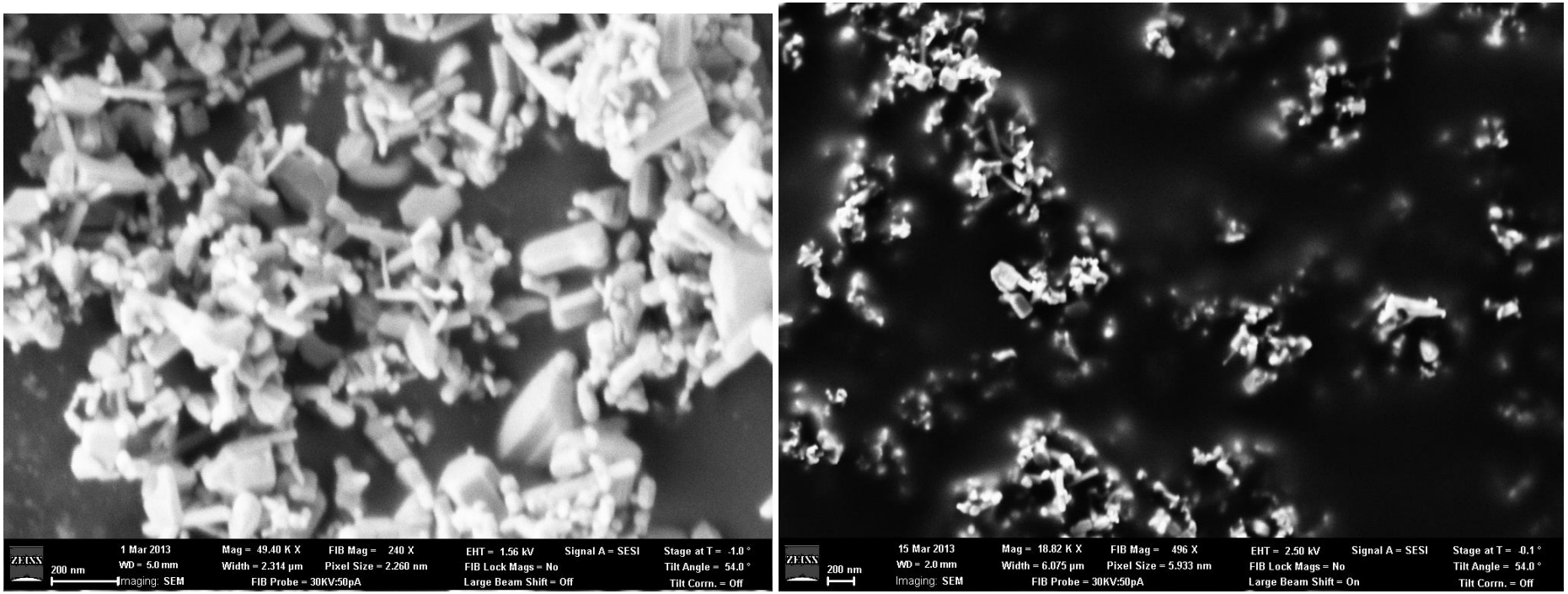
HRSEM of as-obtained Silica nanoparticles without any modification. Aggregation can be evidenced.

Characterization of Nanomaterials



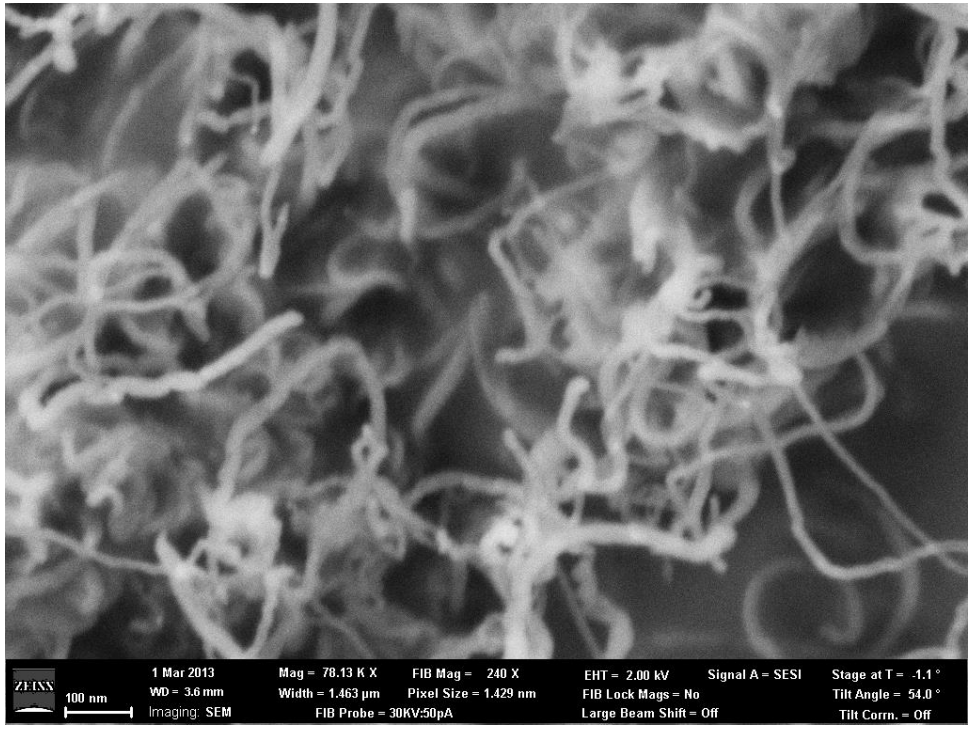
HRSEM of as-obtained Silica nanoparticles modified with (a) Pluronic (left) (b) SDS (right) to control degree of aggregation.

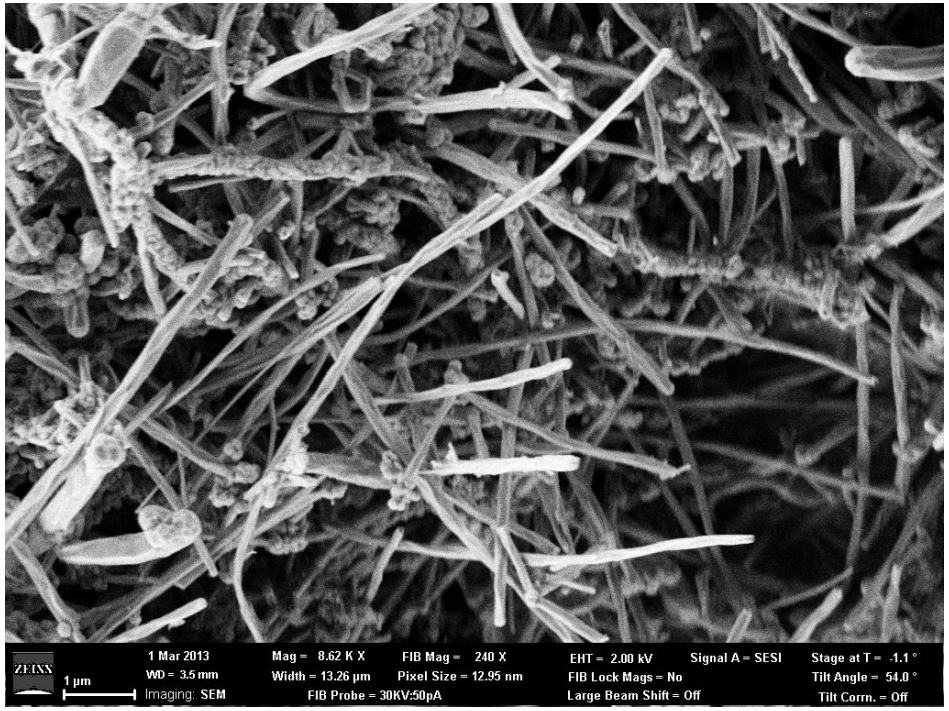
Characterization of Nanomaterials



HRSEM image of ZnO NPs (a) as-obtained without any surface modification and (b) modified with Pluronic. Diameter is less than 100 nm.

Characterization of Nanomaterials

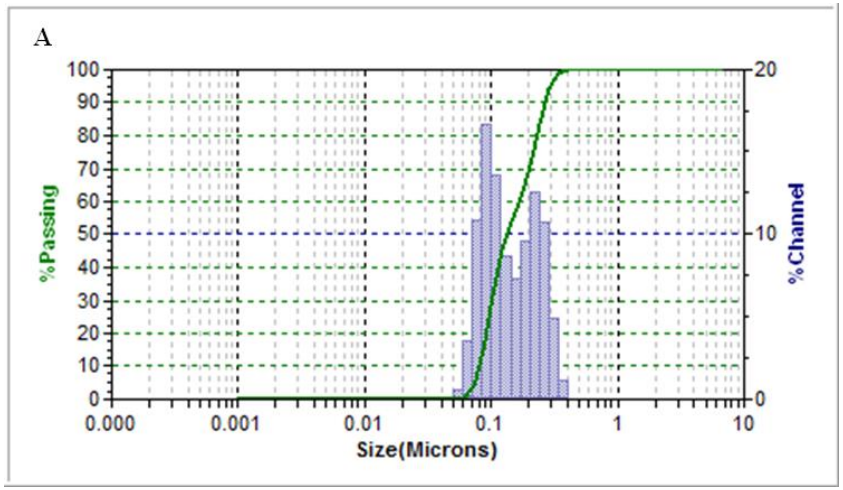


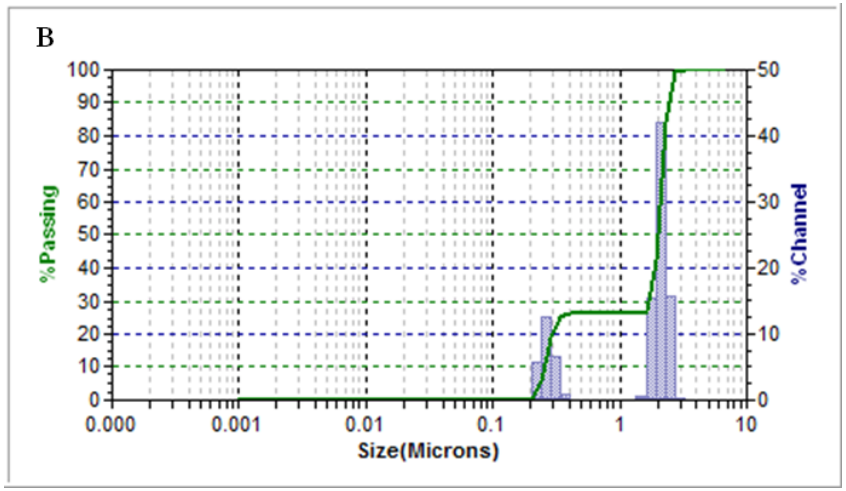


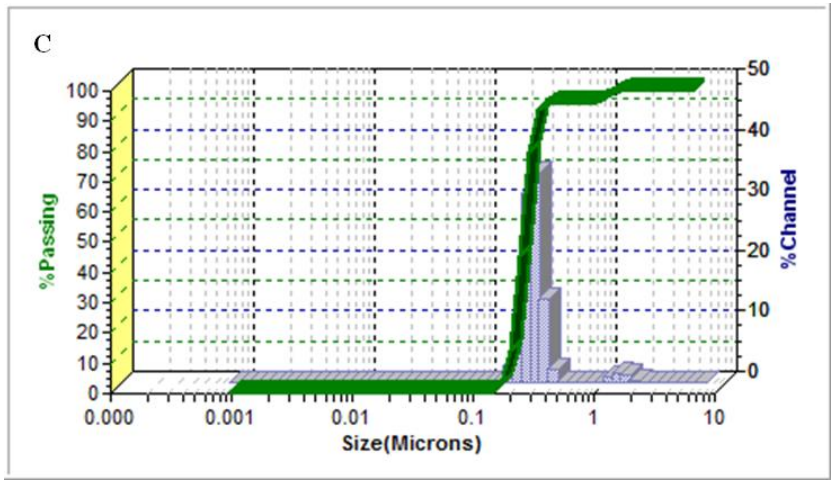
HRSEM of as-obtained short (left) and long (right) MWCNTs without any modification. Large-scale aggregation can be evidenced.

Characterization of Nanomaterials

- **DLS distribution of the starting nanomaterials**



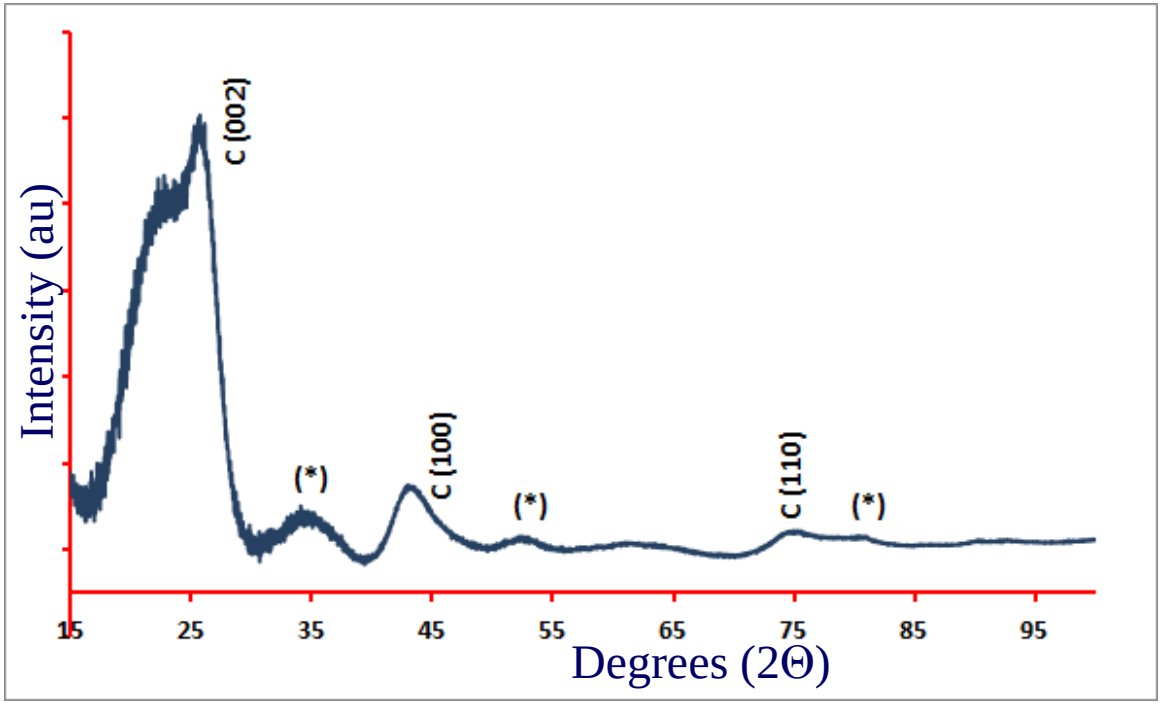




- **DLS data of (A) Au NRs, (B) Silica NPs and (C) ZnO NPs**
- **Increased nanoparticle size is seen in DLS due to particle aggregation.**
- **Initial experiments were done without surface modification of nanoparticles**
- **Currently studying effect of surfactants and resultant aggregation state**

Characterization of Nanomaterials

- **North Carolina A&T/UNC-Greensboro as part of the consortium obtained MWCNT containing powder from UT-Dallas**
- **X-ray diffraction (XRD) patterns were measured with a Oxford Gemini X-Ray Diffractometer with Cu Kα radiation (l = 1.5418 Å)**
- **Graphite like peak (002) is observed along with a family of carbon peaks due to honeycomb lattice of single graphene sheet**



XRD of MWNT containing powder. (*) are metal oxides

Characterization of Nanomaterials

- **Varian 710 ES ICP Axial Spectrometer - Inductively coupled plasma optical emission spectrometry (ICP-OES) used to evaluate and measure metal impurity content in the as-obtained samples**
- **DI water used as control showed no impurities**

Impurity content as measured in ICP-OES

Material	Size (nm)	% Impuriites
Silica	5-15	<0.005
ZnO	<100	<1
MWCNTs	100-170 by 5-9	<0.01
MWCNTs	6-13 by 2.5-20	<0.2

% impurity in MWVNT containing powder obtained from UT Dallas

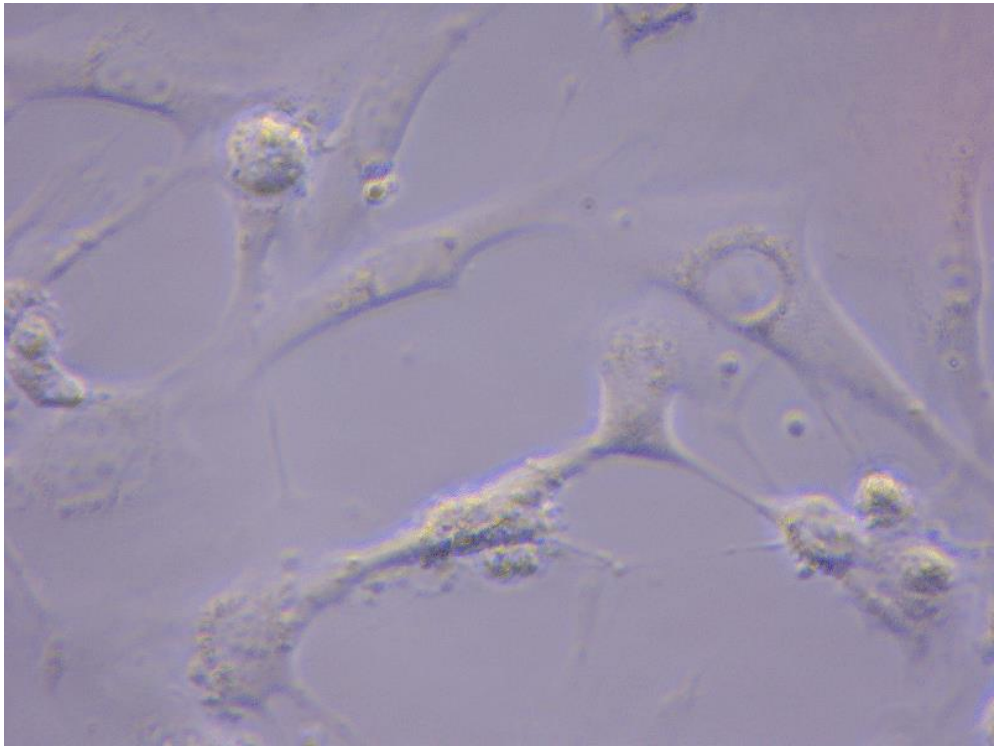
Element	% content
Ni	0.03-0.15
Fe	0.01
Si	0.01-0.07
Al	Nil

- **Trace levels of impurities were measured**
- **Continue to measure size, distribution, structure and composition - Raman Spectroscopy, Nanoparticle Tracking Analysis and Thermogravimetric Analysis**

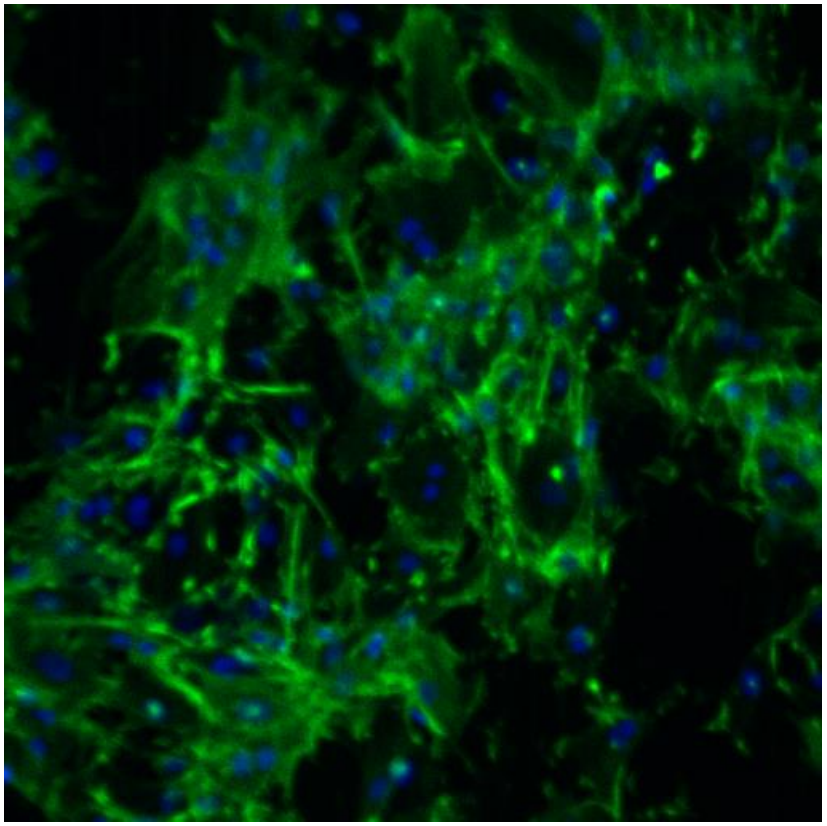
Experimental Methods

- Cytotoxicity assays to study cell membrane integrity and cell viability
- Address two key questions…
  - Does cytotoxicity of ENs vary with cell type?
  - Is cytotoxicity driven by composition, dose and time?
- MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
  - Colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye, MTT, to its insoluble formazan (in viable cells), giving a purple color
- Lactate Dehydrogenase (LDH) assay
  - To assess membrane integrity - by monitoring the passage of substances that are normally sequestered inside cells to the outside.
- Positive and negative controls were incorporated to ensure the assays can detect cytotoxic activity

Cell Morphology

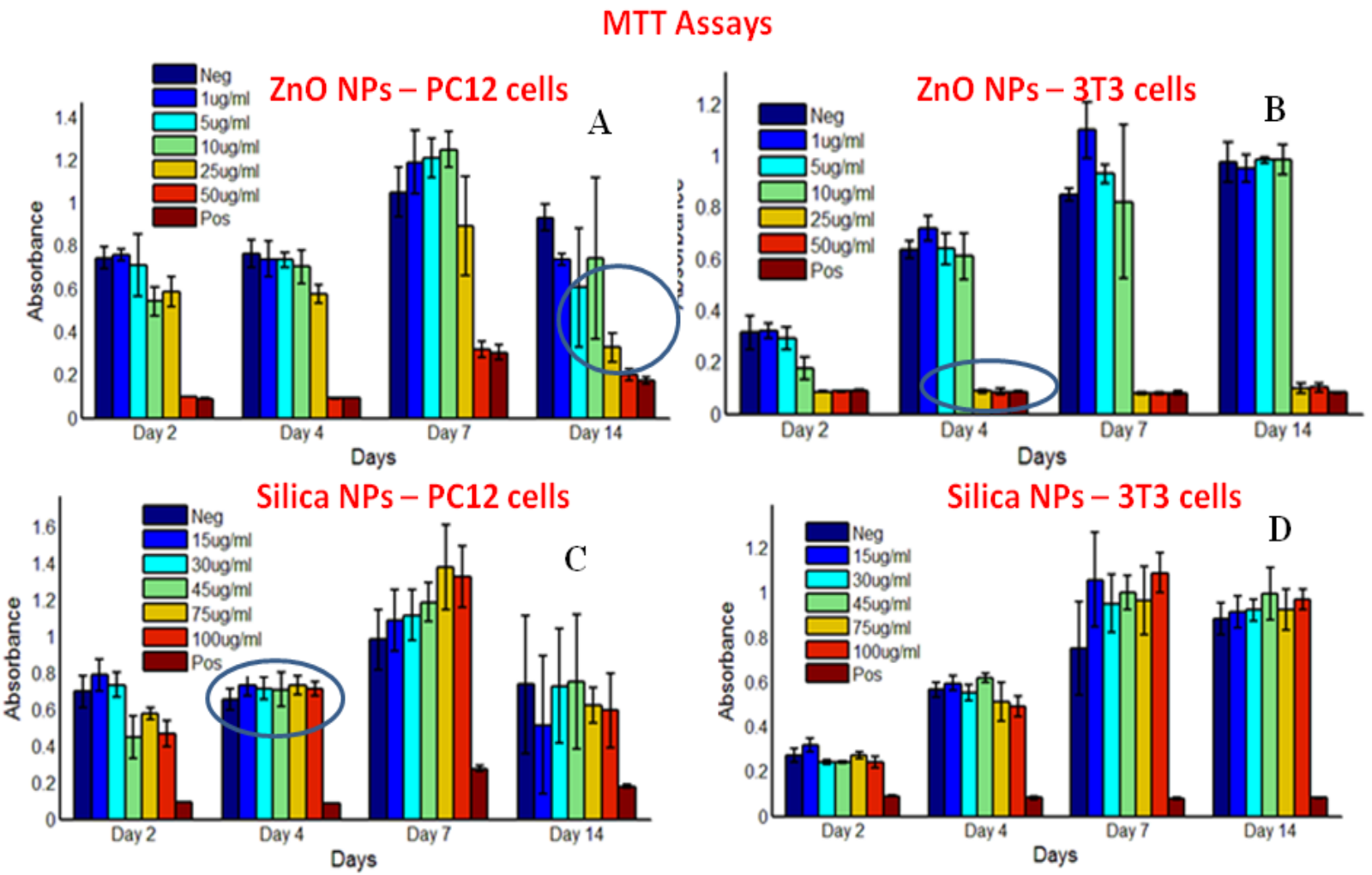


Representative phase-contrast image showing the morphology of NIH/3T3 fibroblastic cells



Representative fluorescence images of nuclei and actin filaments (Molecular Probes, NuCBlue-DAPI, ActinGreen-Alexa Fluor 488)

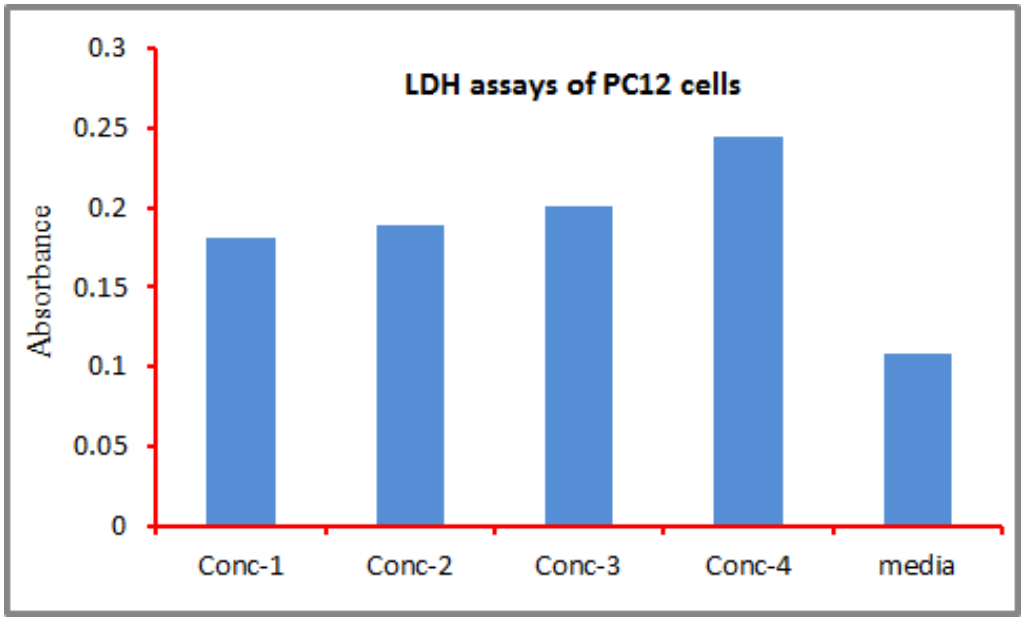
Experimental Results



MTT assays of PC12 cells (A and C) exposed to ZnO and Silica nanoparticles and 3T3 cells (B and D) exposed to ZnO and Silica nanoparticles

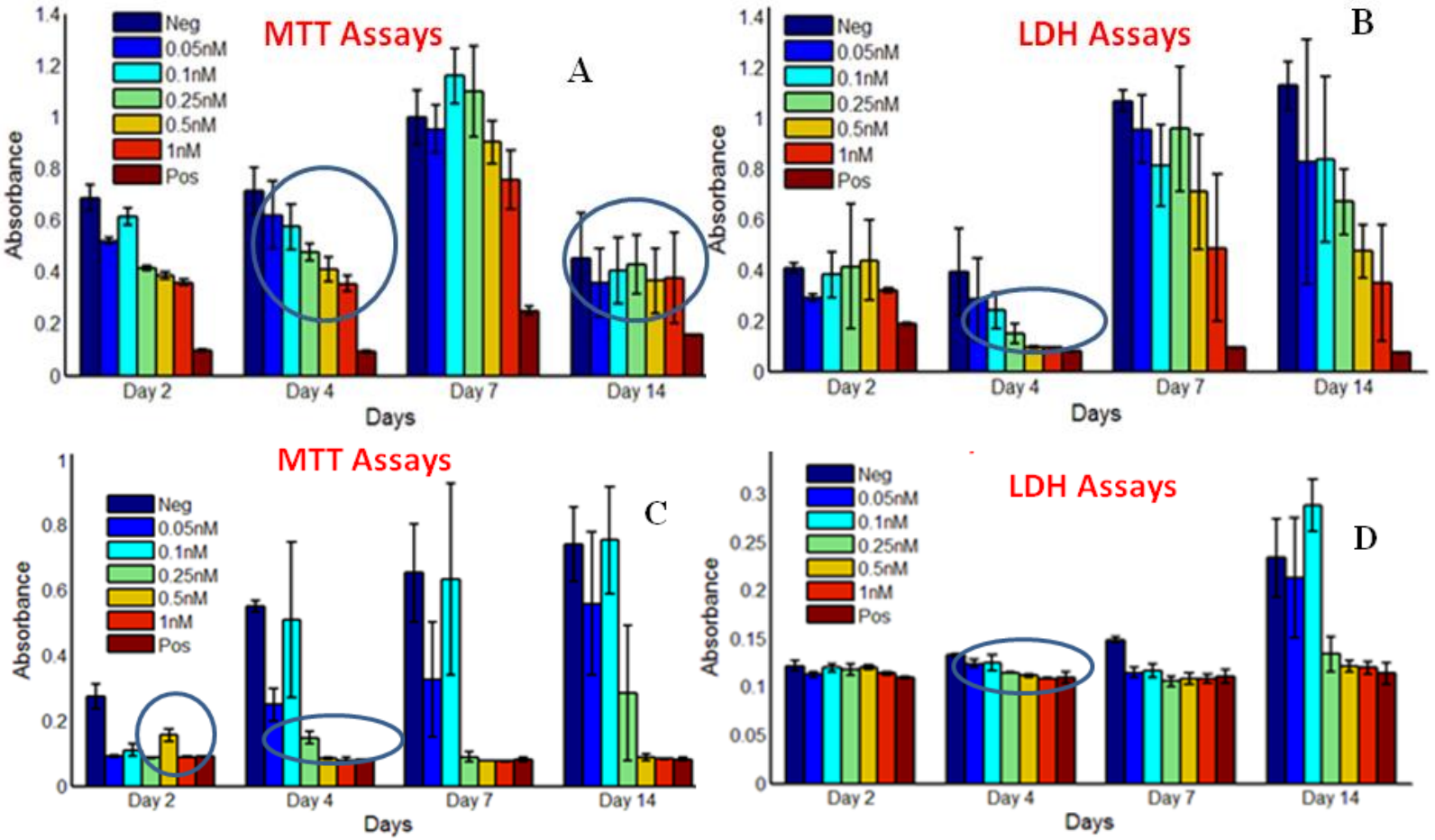
Experimental Results

- **Lactate dehydrogenase (LDH) activity in the cell culture medium was determined by an LDH Kit.**
- **LDH catalyzed the oxidation of lactate to pyruvate with simultaneous reduction of NAD^+ to NADH.**
- **The rate of NAD^+ reduction was directly proportional to LDH activity in the cell medium.**



Calibration of LDH assay of PC12 cells at different cell concentrations. Absorption was measured using a UV–Visible Spectrophotometer at 340 nm

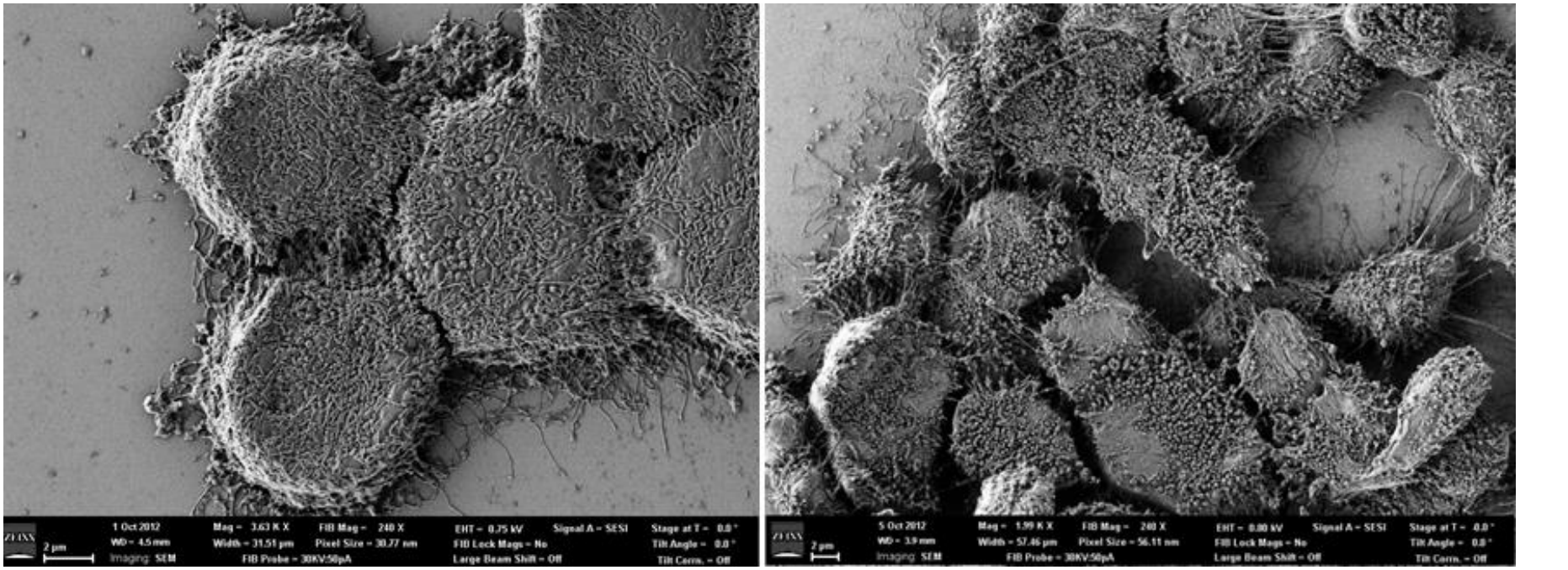
Experimental Results



MTT and LDH assays for Au nanorods exposed to (A and B) PC12 cells seeded at 10000 cells/cm^2 and (C and D) NIH 3T3 fibroblasts seeded at 4000 cells/cm^2

Experimental Results

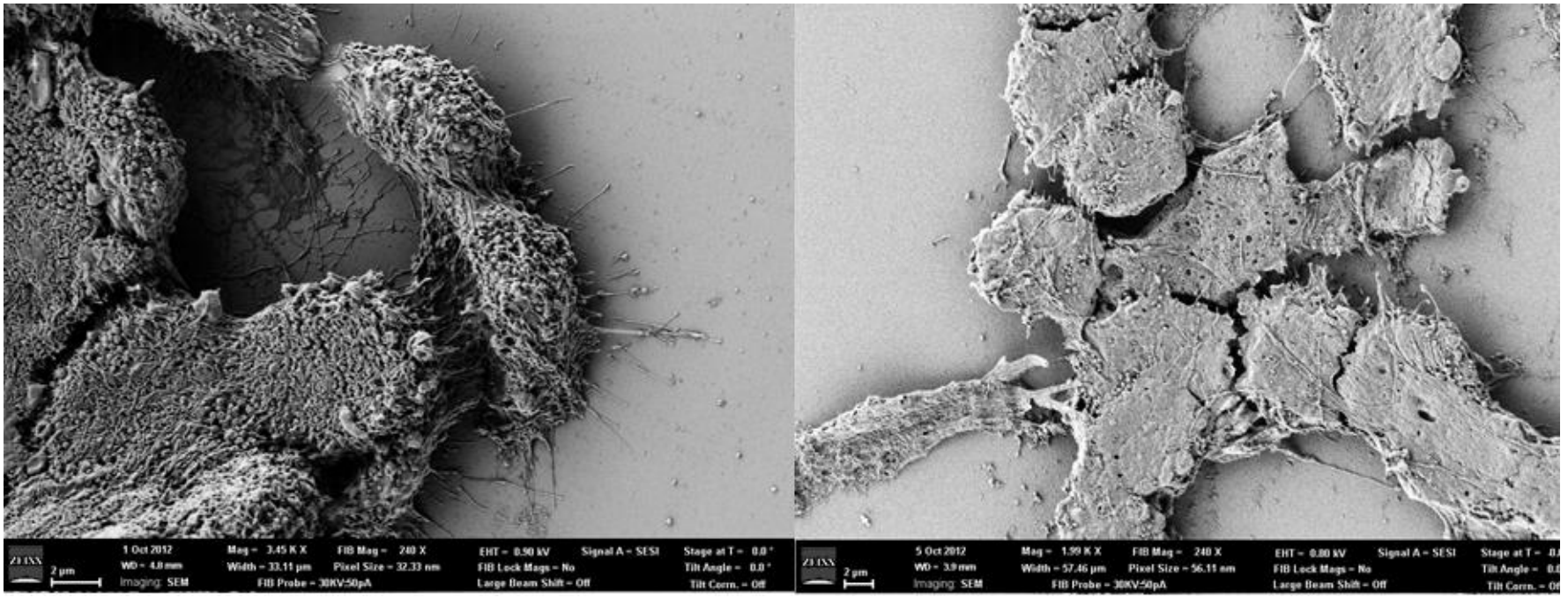
- **Do ENs penetrate into cells and how does intracellular trafficking of ENs occur?**



SEM image of PC12 cells after exposure to 50µg/mL of ZnO nanoparticles at 7 day time point (right), and control (left).

Experimental Results

- **Initial evidence of cell disruption and morphological alterations**

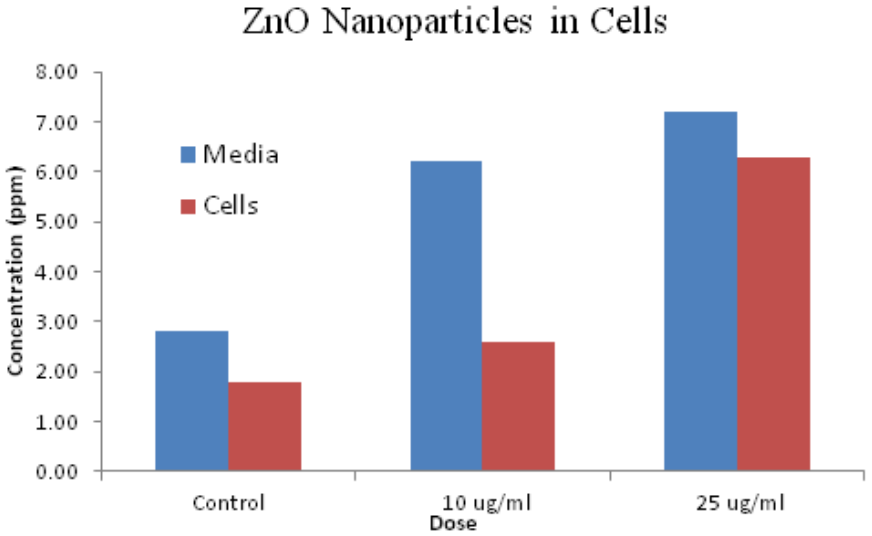


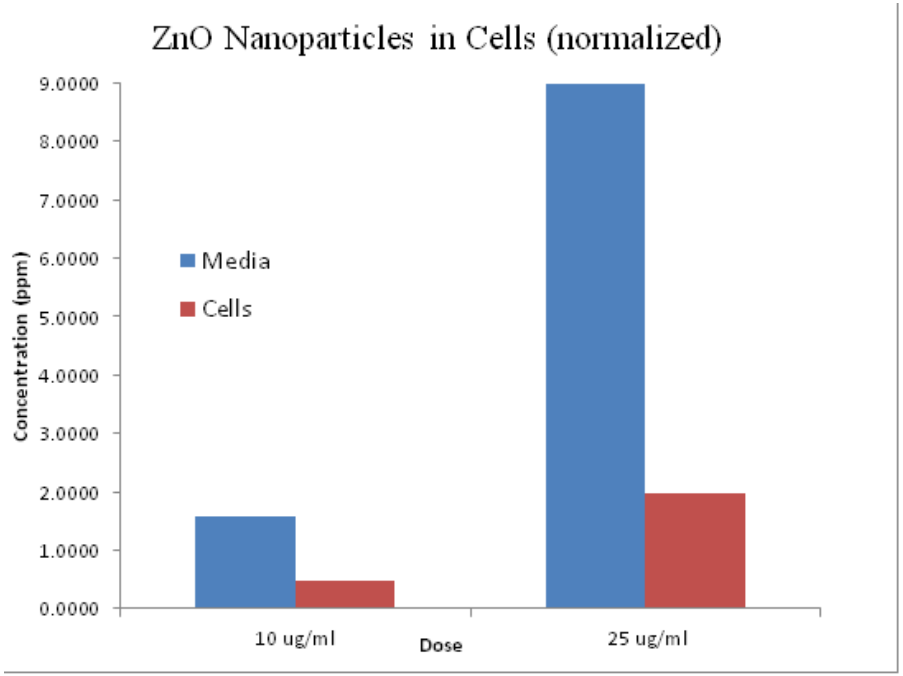
SEM image of PC12 cells exposed to 75 ug/mL of Silica nanoparticles (left) and 0.5nM of Au nanorods (right) at the 7 day time point

- **Currently working on sectioning (ultra-microtome) and ultra-structural characterization and histology (staining of organelles and particles)**

Experimental Results

- **ICP-OES used to study cellular uptake of nanomaterials by evaluating metal content in digested cells**





(Above) Concentration of Zn in media and cells for three ZnO NP doses. (Right) Normalized concentration of ZnO in exposed cells

- **Digestion of cells exposed to ZnO NPs and Au NRs in nitric acid**
- **Measured intensity peaks for Zn and Au in the spectrogram**

Discussion of Results

- **Clear evidence for presence contaminants/impurities in pristine starting nanomaterials. Especially, Si, Fe and Ni in MWCNTs (consortium work)**
- **Increasing dose and time had more pronounced toxic potential, in particular with PC12 cells**
- **Au NRs were seen to be more detrimental to NIH 3T3 compared to PC12 even at lower concentrations**
- **ZnO NPs were more cytotoxic to cells beyond a specific dose – 25µg/mL**
- **Silica NPs (at low dose levels) enhanced the cell growth of both cell types peaking on the 7th day**
- **Morphological alterations to the cell membrane were observed for Au NRs, not pronounced for silica NPs**
- **Clear evidence for cellular uptake of nanomaterials (ZnO and Au) are measured by ICP-OES**

Industrial Interactions and Technology Transfer

- **SRC/ERC Nanotoxicity consortium meetings - Discussed research needs with academia and industrial members**
  - **Conduct round-robin studies with other consortium members on CNTs and CMP materials such as CeO**
- **Hosted Teleseminars for SRC/ERC for Environmentally Benign Semiconductor Manufacturing on May 31st 2012 and Sept 6th 2012**

Future Plans

Next Year Plans

- Continue with nanomaterials decided by consortium – MWCNTs (long, short), CeO and other CMP materials – Al_2O_3, Silica, ZnO
- Complete comprehensive starting material characterization including DLS, XRD, SEM, TEM, ICP, Raman, FTIR, TGA
- Relevant cell types in the respiratory track – human derived cells especially A549 lung epithelial cells and macrophages
- Quantify cell uptake and long-term toxicity
  - Using longitudinal electrical impedance spectroscopy (EIS)-on chip and analytical and microscopy (ICP-OES, HeIM, TEM, Confocal)
- Cyto/Genotoxic assay- Live-cell imaging, ROS and DNA damage

Long-Term Plans

- Validation of non-conventional techniques to assess toxicity
- Dose-response relationship for different physiochemical ENs
- Mechanism of cellular uptake (track cell behavior) and toxicity in order to safely design and use nanomaterials

Publications, Presentations, and Recognitions/Awards

- **S. Aravamudhan, Invited Talk at Nano@Tech, Georgia Institute of Technology, Oct 2012**
- **K. Garde, S. Crawford, S. Aravamudhan, "Understanding Cytotoxicity of Engineered Nanomaterials," ECS Transactions, 50, 2013**
- **K. Garde, S. Aravamudhan, "Toxicity Assay-on-Chip for Engineered Nanomaterials," 2012 Electrochemical Society Meeting**
- **S. Aravamudhan, K. Garde, S. Crawford, K. Kosaraju, "Toxicity Models and Platforms for Engineered Nanomaterials," Chapter in Advances in Nanoscience and Nanoengineering, CRC Press (Tentatively to be published in late 2013)**
- **K. Garde, S. Woosley, S. Crawford, S. Aravamudhan, "Towards Tracking Cellular Response and Understanding Cytotoxicity of Engineered Nanomaterials," TECHCON 2013 (submitted)**