Studying the cellular uptake and toxicity of CMP NPs

ERC Teleseminar 6/5/2014

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Nanoscience and Nanoengineering

Review - SRC Annual Review Meeting

During the SRC review meeting we mainly discussed-

- ✓ Characterization of slurries using ICP-OES, FT-IR, XRD, DLS, Zeta Potential, SEM and TEM.

- ✓ Effect of slurries on A549 cell viability, toxicity and membrane integrity.
  - ❑ Slurry 1 and slurry 2 exhibited toxicity

- ✓ Effect of supernatants on A549 cel viability, toxicity and membrane integrity.
  - ❑ Supernatant from slurry 2 showed toxicity

Focus of the presentation

- Observations from cell viability and cytoxicity experiments.

- Understanding the cellualr uptake of slurries.
  - Analytical Techniques: Confocal Raman, ICP-OES and ECIS.
  - Biochemical method: ROS.

Introduction

- Engineered Nanomaterials (EN) have been proven to have tremendous potential in various fields like health care, energy, environmental science, food safety, information technology, transportation and many others.
- Along with the tremendous potential EN offer, it has become evident that they can have a potential impact on environment, health and safety especially the toxicity to the environment when they are used at a large scale.
- Understanding the uptake of EN by the cells is an important aspect that needs serious attention.

Experimental Pathway

I

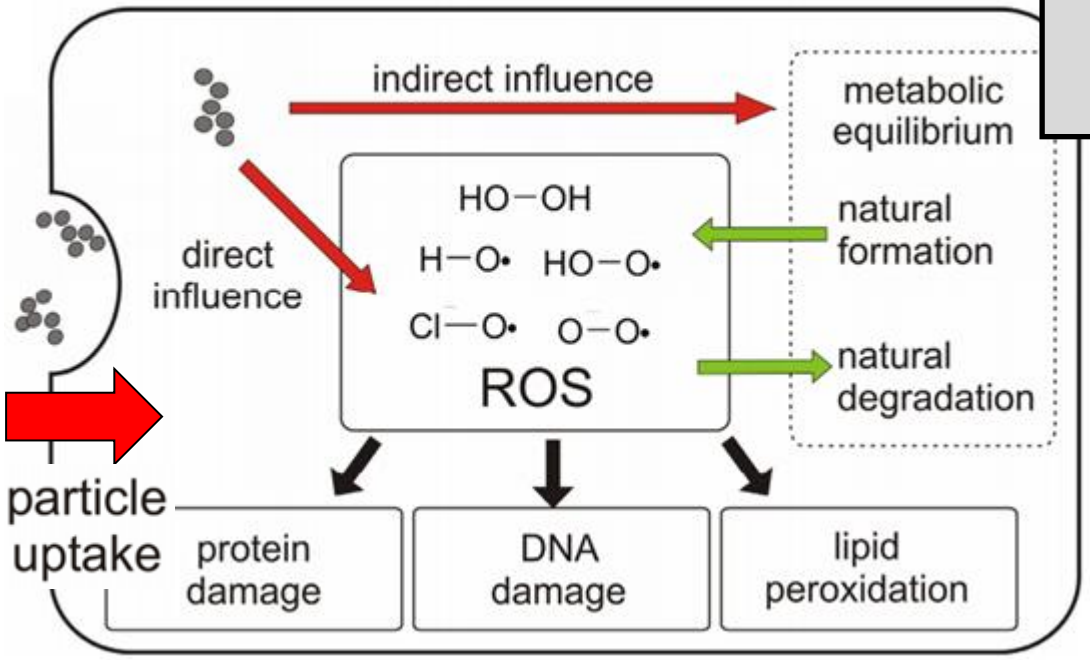
Comprehensive Characterization of ENs

(DLS, Zeta potential, BET, FTIR, XRD, Raman, ICP-OES, HRSEM, TEM)

High-Content Assays/Longitudinal Methods

II

Delayed cytotoxicity (cell viability and membrane integrity) using MTT and LDH assays



Safe EN design, Predictive nanotoxicity

IV

Delayed genotoxicity studies – Comet assays

III

Acute/delayed cellular uptake and internalization

(ICP-OES, Confocal Raman, ECIS, TEM, ROS)

Nanoparticles and Cells

- Nanoparticles (NPs) and NP slurries investigated

Sample	Composition	pH	Size (nm)
Colloidal Silica (NS-0813-01)	3% precipitated silica; adjusted with acetic acid	2.5-4.5	50-60
Fumed Silica (NS-0813-02)	5% silica; adjusted with KOH	10	120-140
Ceria (NS-0813-03)	1% Ceria;	3-4	60-100
Alumina (NS-0813-04)	3% Alumina; adjusted with nitric acid	4.5-5	80-100

- Cells studied- A549 adenocarcinomic human alveolar basal (lung) epithelial cells

Presentation Outline

I. Cytotoxicity of NPs (MTT and LDH assay)

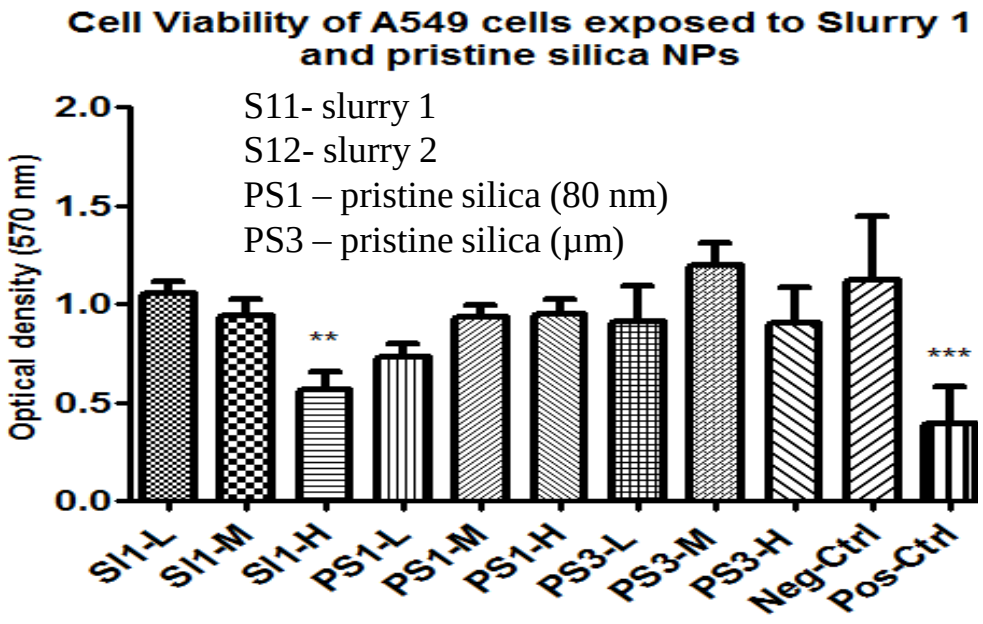
II. Uptake and internalization of NPs (ICP-OES, Confocal Raman, ECIS and ROS)

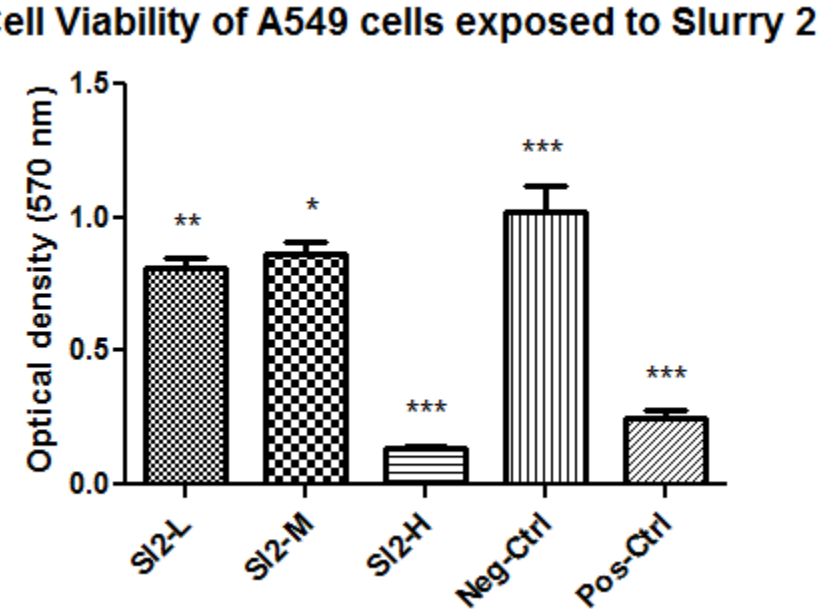
I. Cytotoxicity – Cell Viability and Integrity

Sample		Volume of slurry (in µL)	Concentration (mg/mL)	pH
Slurry 1	High (H)	10	2.03	7.55
	Medium (M)	1	0.203	7.74
	Low (L)	0.1	0.0203	7.78
Slurry 2	High (H)	10	3.34	8.2
	Medium (M)	1	0.334	7.83
	Low (L)	0.1	0.0334	7.76
Slurry 3	High (H)	10	0.52	7.7
	Medium (M)	1	0.052	7.74
	Low (L)	0.1	0.0052	7.73
Slurry 4	High (M)	10	2.01	7.72
	Medium (M)	1	0.201	7.81
	Low (L)	0.1	0.0201	7.85

I. Cytotoxicity – Cell Viability and Integrity

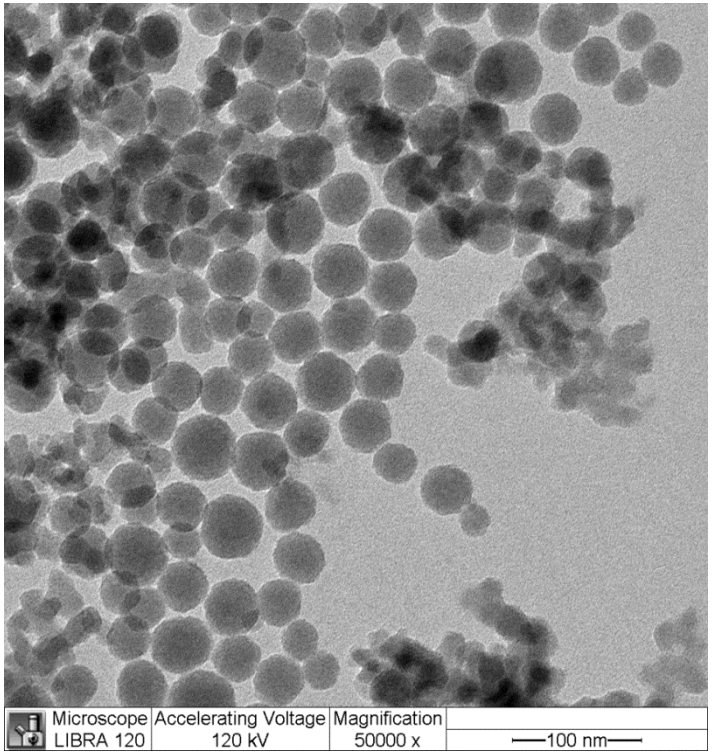
- A549 cells (density of 10,000/cm^2) exposed to specified amount of slurries and same concentration of pristine NPs for 48 hours; the assay is performed after another 24 hours
- Absorbance measured at 570 nm
- ANOVA (Analysis of Variance) - "one-way ANOVA" using a significance level of 0.05 (95% confidence intervals); *** - 99.9% confidence interval; ** - 99% confidence and * - 95% confidence interval



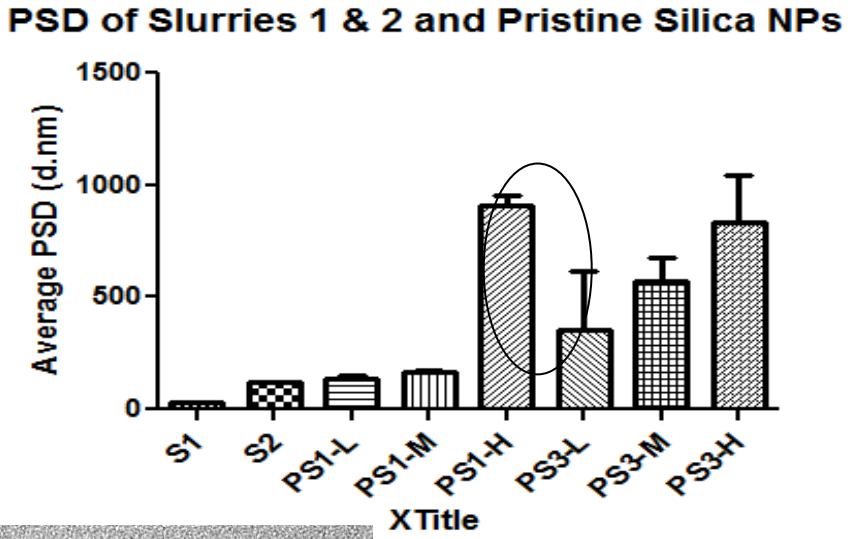


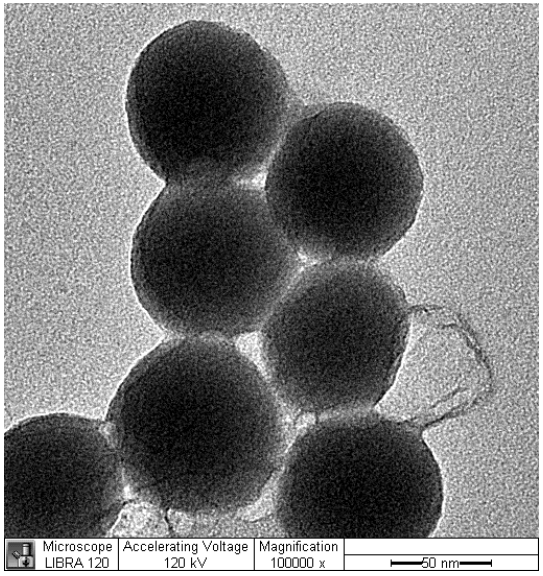
I. Cytotoxicity – Cell Viability and Integrity

- Aggregation of colloidal silica NPs in slurries and pristine colloidal silica NPs



TEM of Colloidal Silica NPs in Slurry 1



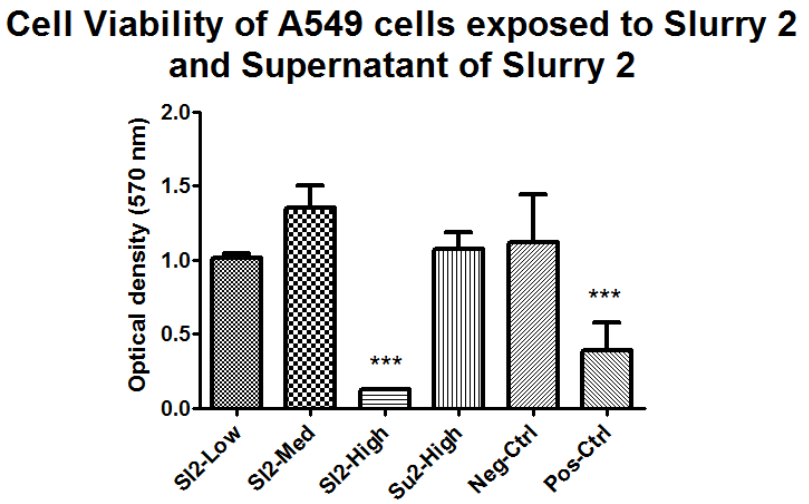


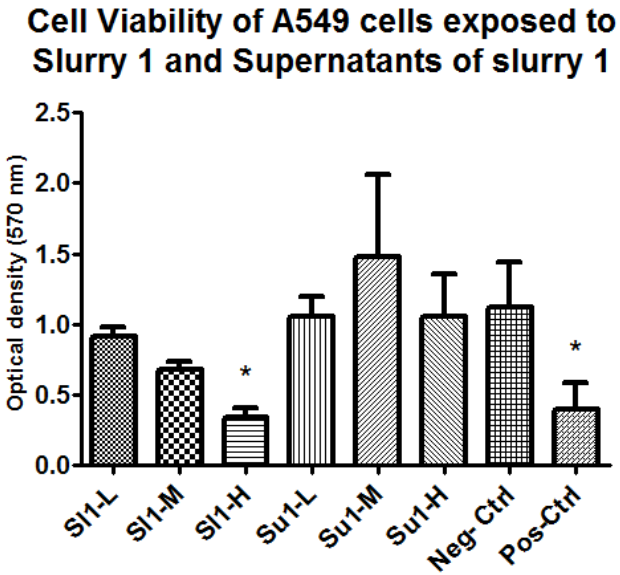
PS1 – pristine colloidal silica (80 nm)
PS3 – pristine colloidal silica (200 nm)

TEM of Pristine Colloidal Silica NPs of same size as silica in slurry 1

I. Cytotoxicity – Cell Viability and Integrity

- Effect of slurry supernatants on A549 cell viability
- Supernatants were prepared by UTD by removing nanoparticles by centrifugation at 200,000g
- As stated earlier, the same MTT cell viability procedures were followed



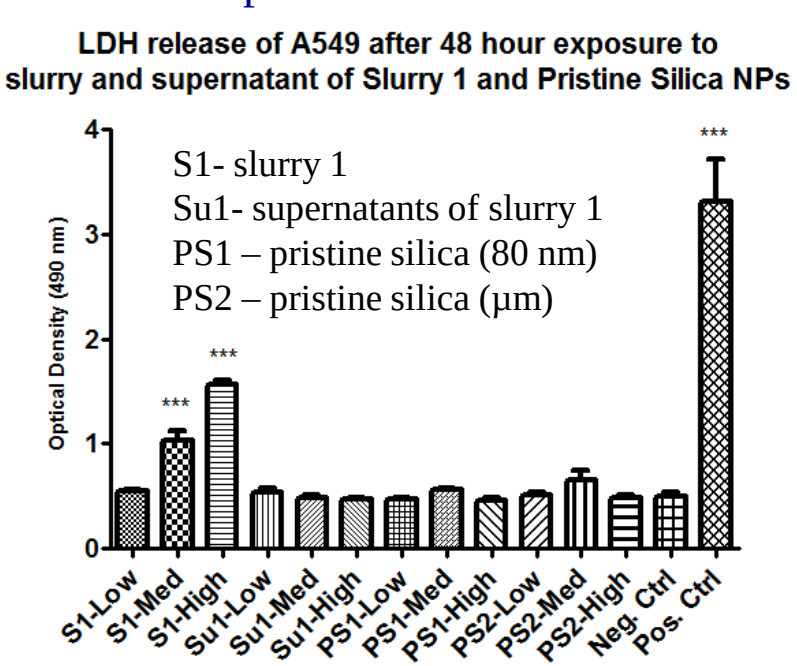


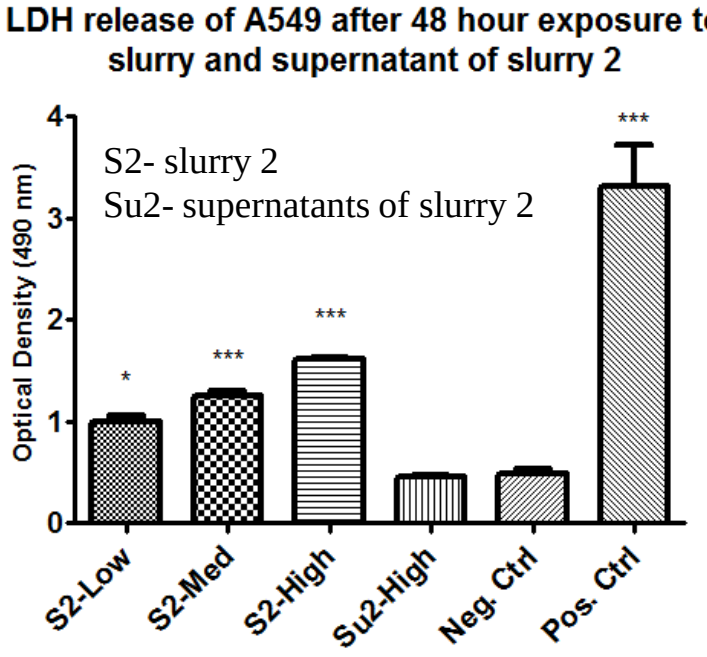
Su1- supernatant of slurry 1
Su2- supernatant of slurry 2
Su3- supernatant of slurry 3
Su4- supernatant of slurry 4

- Slurry supernatants showed no to minimal cytotoxicity, compared to control cells

I. Cytotoxicity – Cell Viability and Integrity

- Lactate Dehydrogenase (LDH) assay
  - Assessment of membrane integrity - by monitoring the passage of substances that are normally sequestered inside cells to outside
- LDH assay was performed after 48 hours of NP exposure using the supernatants from the well plate

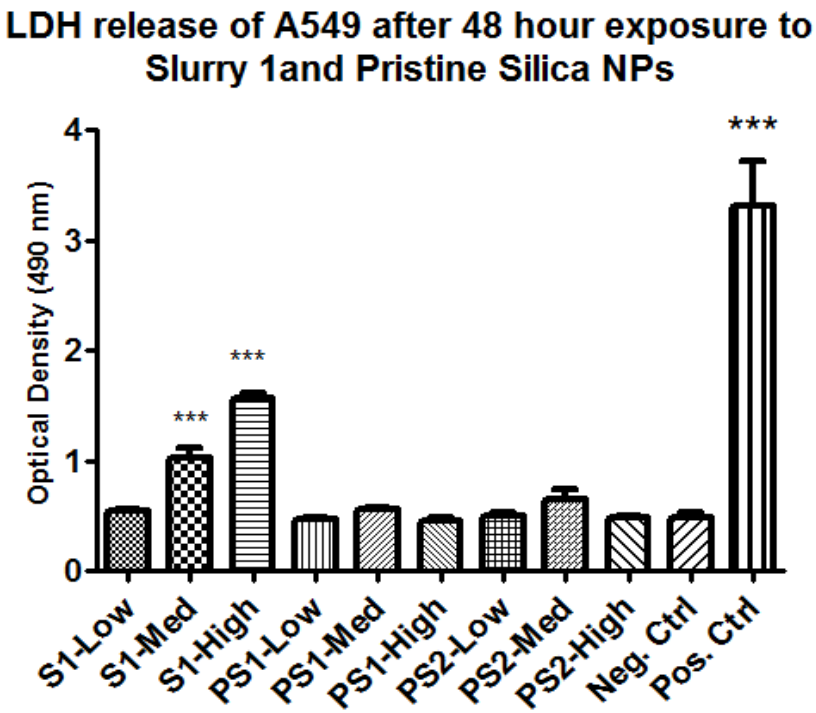


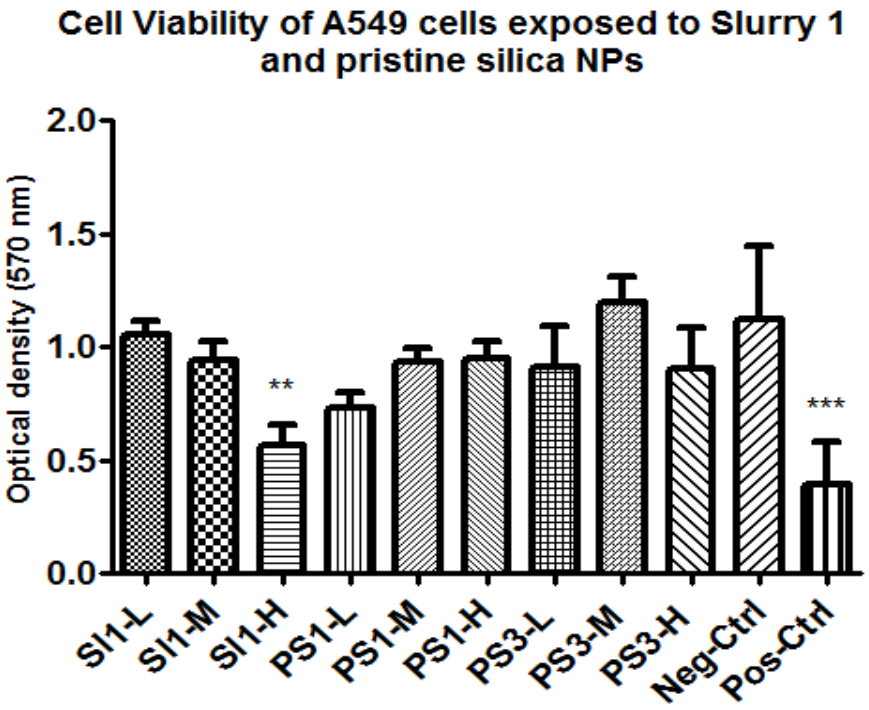


- Similar to MTT assays, silica NP slurries showed a dose-dependent LDH response, compared to control cells and pristine micro and nanoparticles

I. Cytotoxicity – Cell Viability and Integrity

- Comparison of MTT and LDH assay for slurry 1 and colloidal pristine silica micro and nanoparticles
- Excellent correlation among the two cell viability and membrane integrity assays





S1/S11 – slurry 1, PS1 – pristine silica (80 nm), PS2/PS3 – pristine silica (µm)

II. Cellular Uptake and Internalization

- Do ENs penetrate into cells? If so, where do they accumulate or internalize? What about Reactive Oxygen Species (ROS)?
- Studied cell uptake and internalization
  - Inductively coupled plasma optical emission spectrometry (ICP-OES)
  - Confocal Raman Microscopy
  - Electrochemical Cell Impedance Spectroscopy (ECIS)

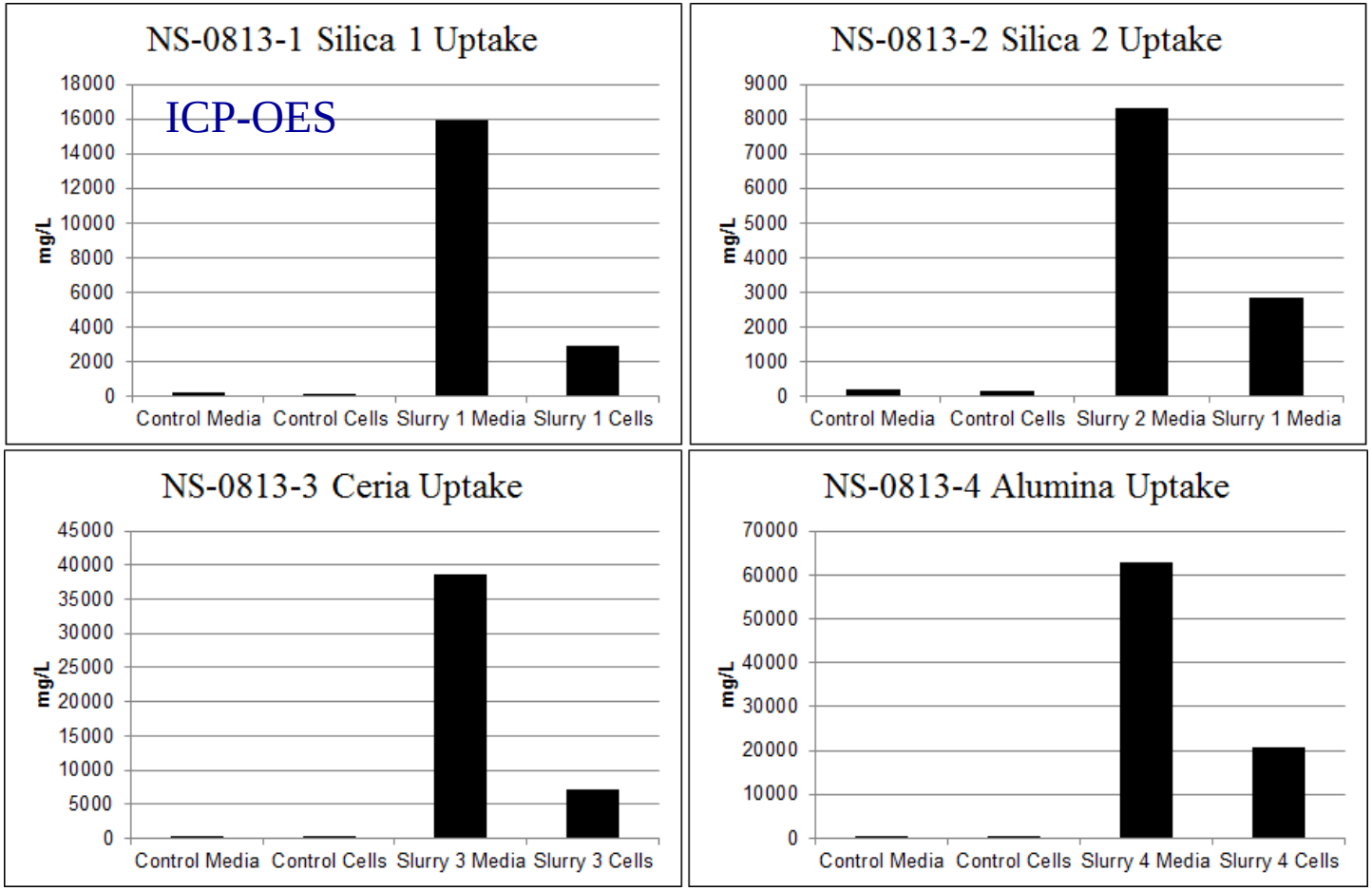
II. Cellular Uptake and Internalization

ICP-OES is an analytical technique used for the detection of trace metals

- Intensity of the emission is indicative of the concentration of the element within the sample
- Same exposure conditions were followed as in MTT/LDH, highest concentration of slurries were chosen
- Before analysis, cells were washed and cells exposed to the slurries were then digested as per standard protocols

Samples	Concentration (mg/mL)
Slurry 1	2.03
Slurry 2	3.34
Slurry 3	0.52
Slurry 4	2.01

II. Cellular Uptake and Internalization

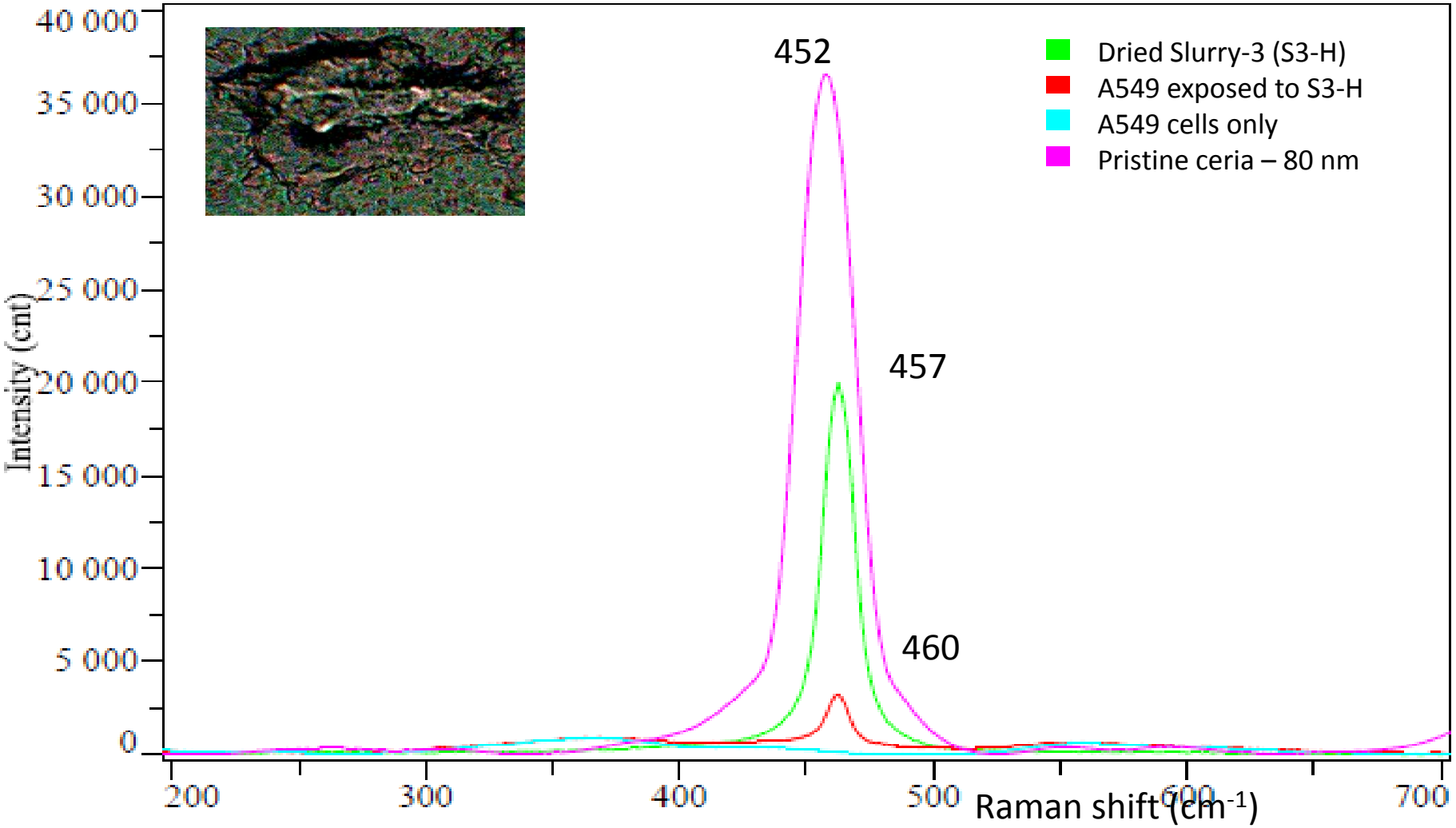


- All NP slurries (high concentration) showed some amount of cellular uptake compared to controls, but just uptake does not necessarily lead to toxicity.

II. Cellular Uptake and Internalization

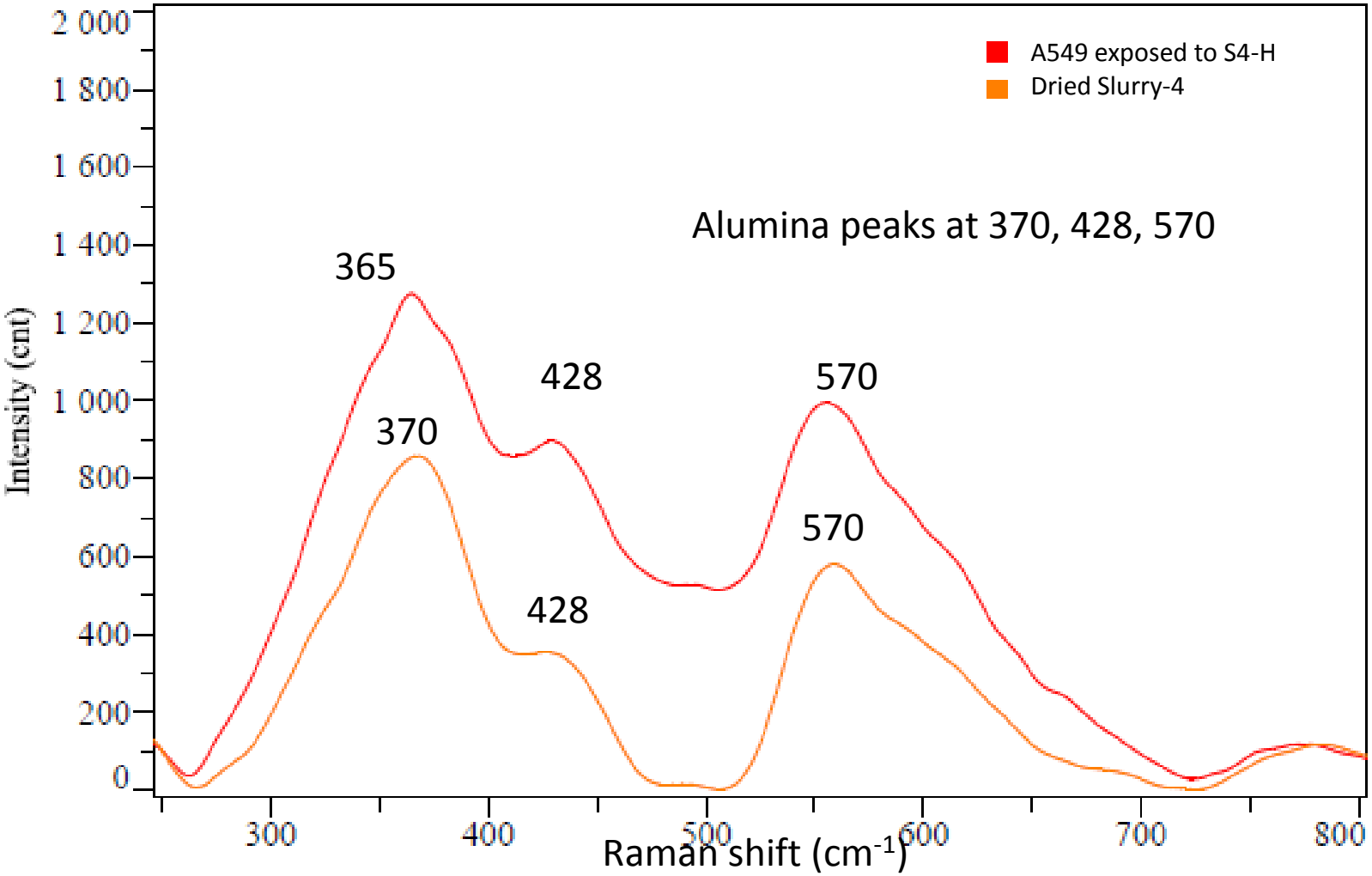
- Confocal Raman, a non-invasive, non-destructive and label-free technique, was employed to study uptake and localization of NPs
- Horiba XploRA Raman Confocal Microscope System; Spot size – 1.12 µm
- NPs show Raman active vibration modes
- Ceria NPs - a single strong band located around 450-465 cm^{-1}
- For alumina NPs, from the 27 possible optical modes in the crystal structure, 7 modes are Raman active and 6 modes are infrared (IR) active
- Same A549 cell exposure conditions were followed as in MTT/LDH, highest concentration of slurries were chosen
- Before analysis, cells were washed with PBS several times to remove surface bound NPs.

II. Cellular Uptake and Internalization



- Clear evidence of Ceria NP localization within A549 cells

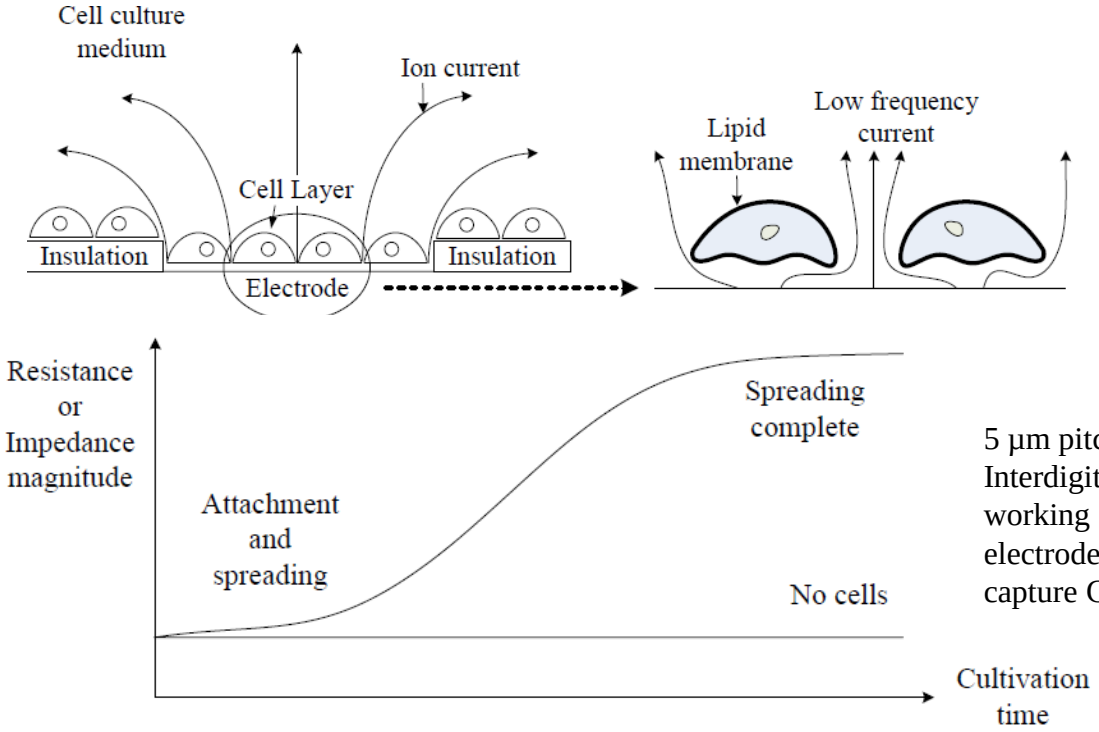
II. Cellular Uptake and Internalization



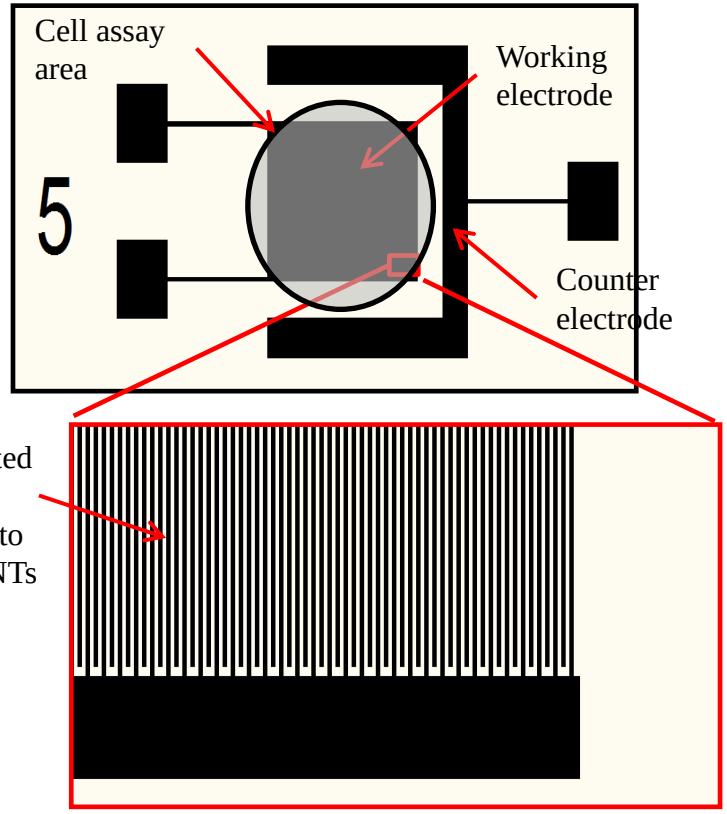
- Evidence of Alumina NP localization within A549 cells

II. Cellular Uptake and Internalization

- Development and optimization of a high-content screening/monitoring method – Electrochemical Cell Impedance Spectroscopy (ECIS)



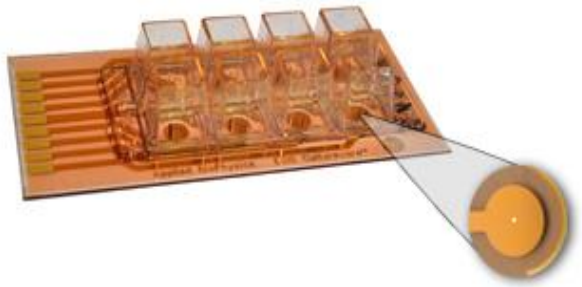
Schematic of microelectrode-based cell chip, below: impedance recorded at the frequency of 4 kHz during the cell growth on the electrode (Giaever and Keese 1993)



Design to monitor cell impedance

II. Cellular Uptake and Internalization

ECIS measurements (Real Time)



- Performed using 8W1E electrode arrays purchased from Applied Biophysics.

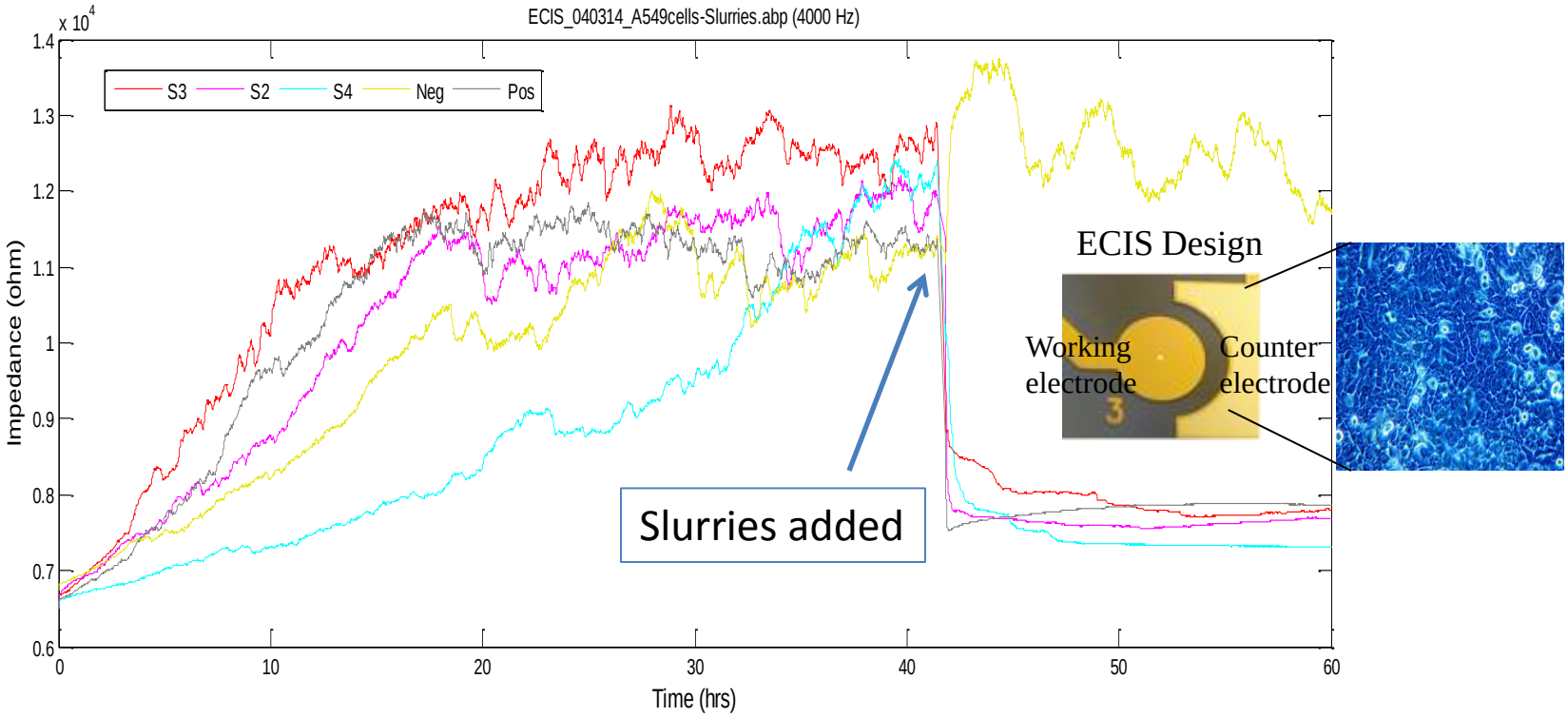
- Each of the 8 wells contains a single circular 250μm diameter active electrode with an area of 0.8 cm^2 . On average, with a confluent cell layer, approximately 50 to 100 cells will be measured by the electrode, but even a single cell can be observed.

- Cells were seeded at a density of 10, 000 cells/cm^2. After attachment, impedance measurements were performed using a ECIS Zθ instrument. The slurries were added after cell spreading on the electrodes is complete.

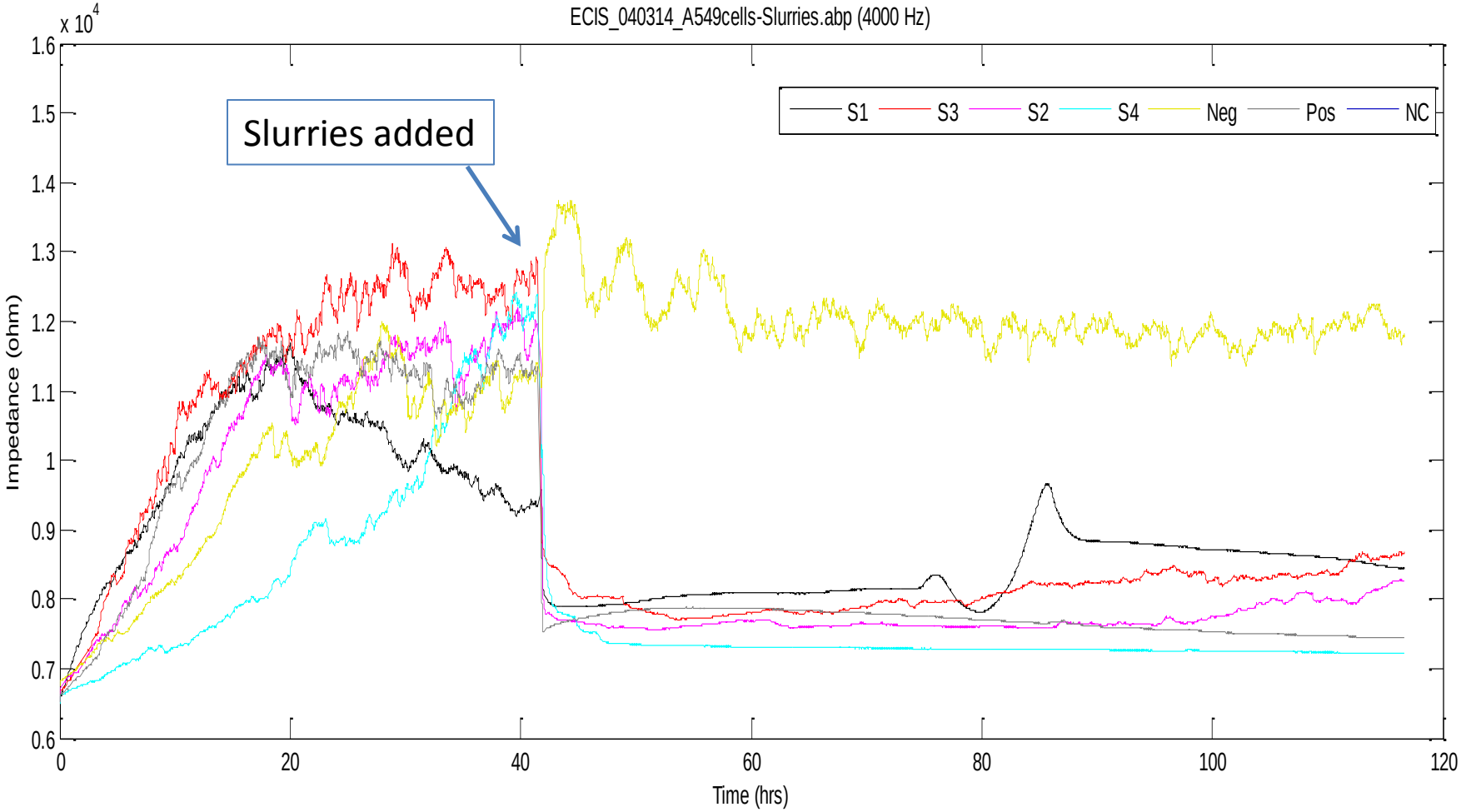
II. Cellular Uptake and Internalization

ECIS data for A549 cells exposed to slurries measured at 4 KHz using ECIS 8W1E electrode arrays purchased from Applied Biophysics.

Concentrations : Slurry 2 - 3.34 mg/mL, Slurry 3 - 0.52 mg/mL and Slurry 4 - 2.01 mg/mL



II. Cellular Uptake and Internalization



II. Cellular Uptake and Internalization

ROS release:

- A549 cells at a density of 10, 000 cells/cm^2 were seeded in a 96 well plate.
- 24 hours after seeding, the cells were exposed to 20 uM Carboxy-H2DCFDA, purchased form Life Technologies, for 45 minutes and then exposed to slurries for 6, 12 ,24 and 48 hours.
- The fluorescence was recorded at ex/em wavelengths of 480/520 nm at 37 degrees using plate reader at respective time intervals.

II. Cellular Uptake and Internalization

Sample		Volume of slurry (in µL)	Concentration (mg/mL)	pH
Slurry 1	High (H)	10	2.03	7.55
	Medium (M)	1	0.203	7.74
	Low (L)	0.1	0.0203	7.78
Slurry 2	High (H)	10	3.34	8.2
	Medium (M)	1	0.334	7.83
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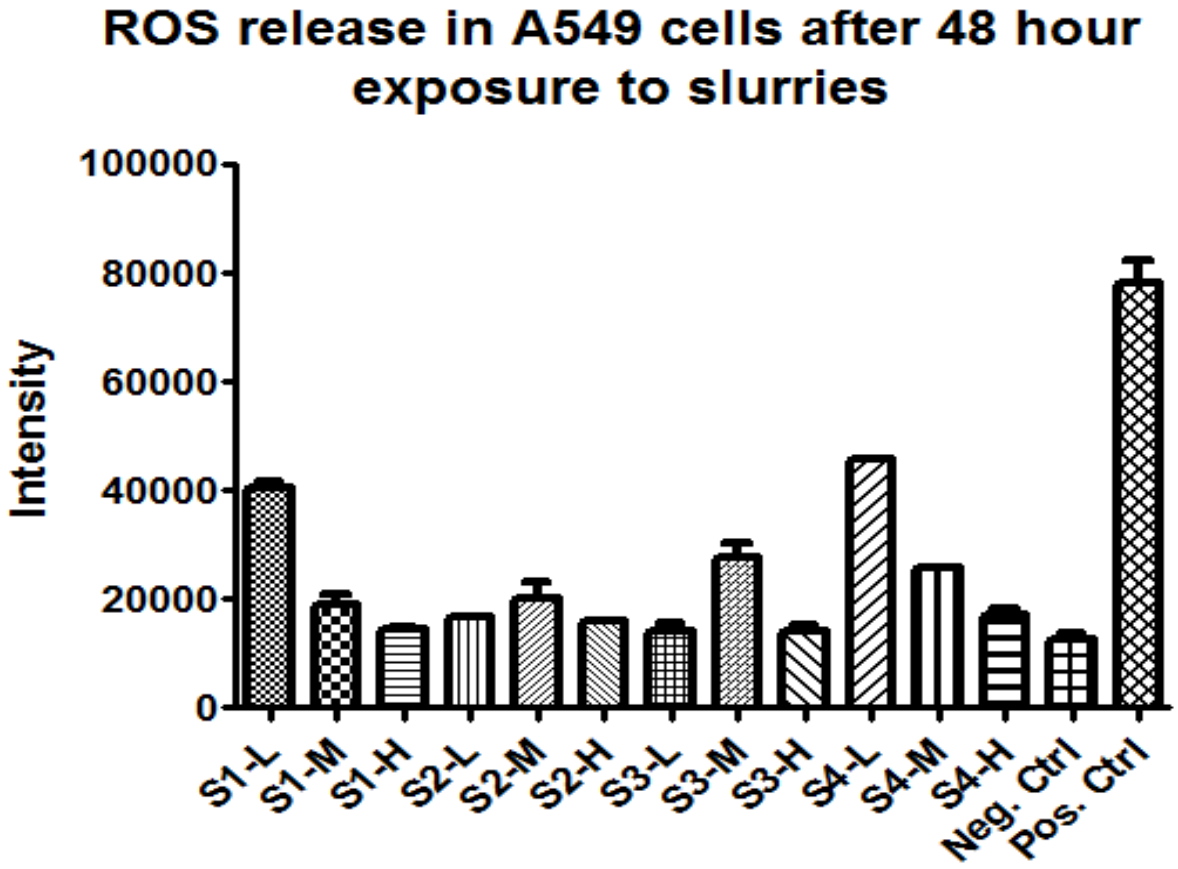
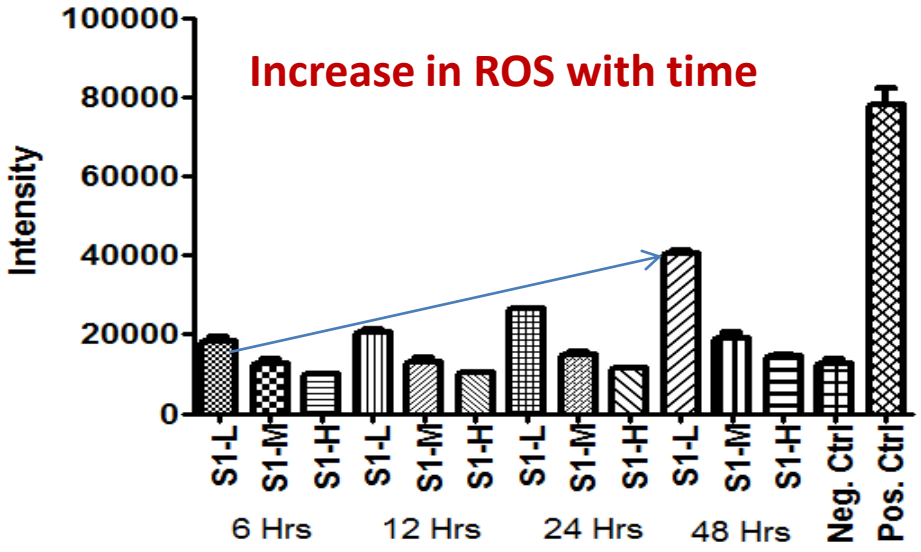
II. Cellular Uptake and Internalization

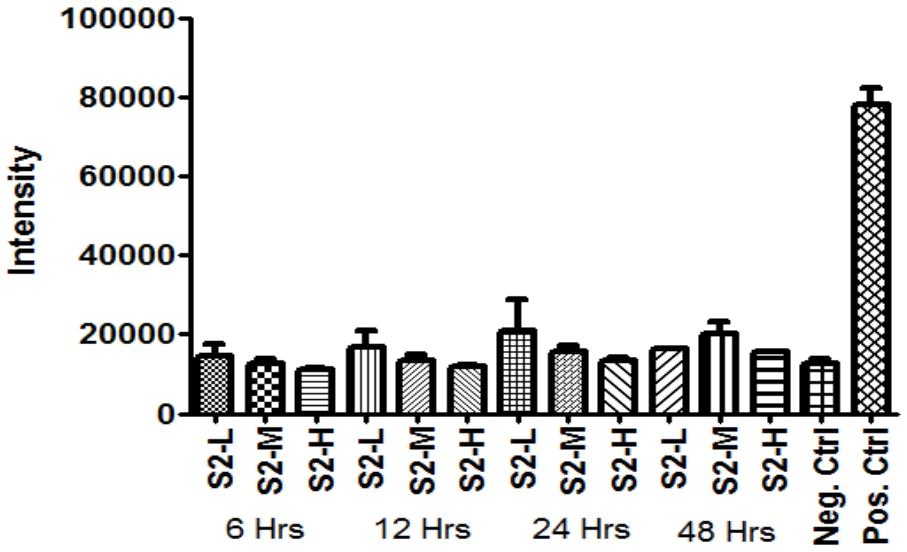


- A549 cells exposed to low concentrations of slurry 1 and slurry 4 showed relatively high ROS production. But, slurries 2 and 3 show minimal to no ROS production

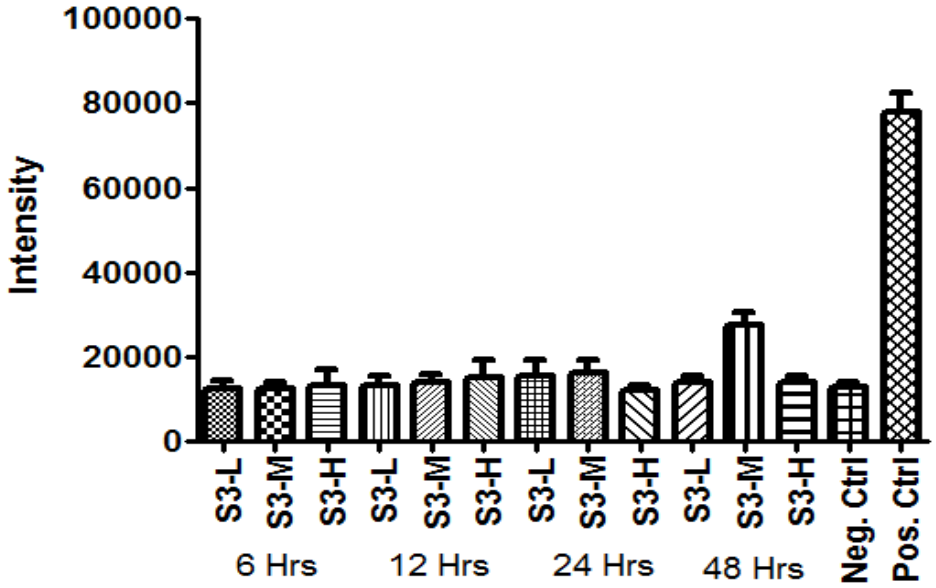
ROS release in A549 cells on exposure to slurry 1



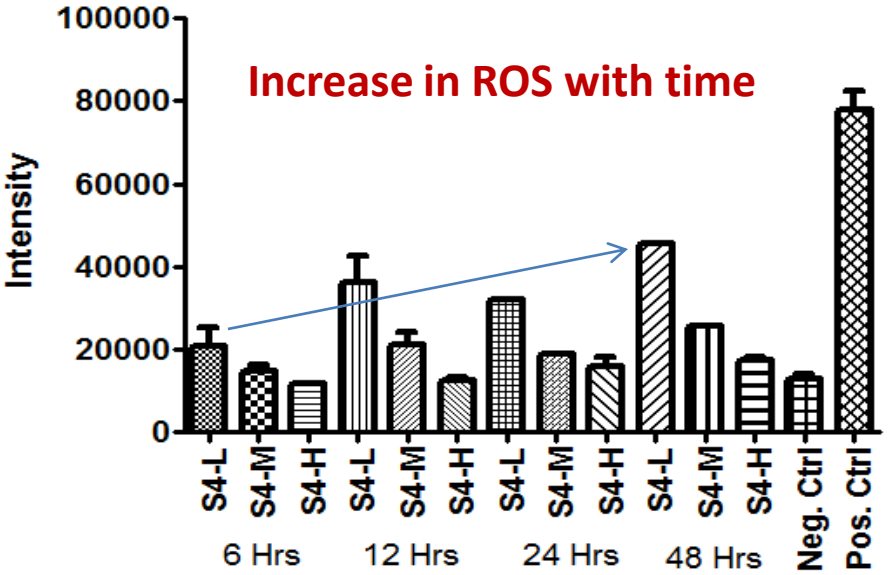
ROS release in A549 cells on exposure to slurry 2



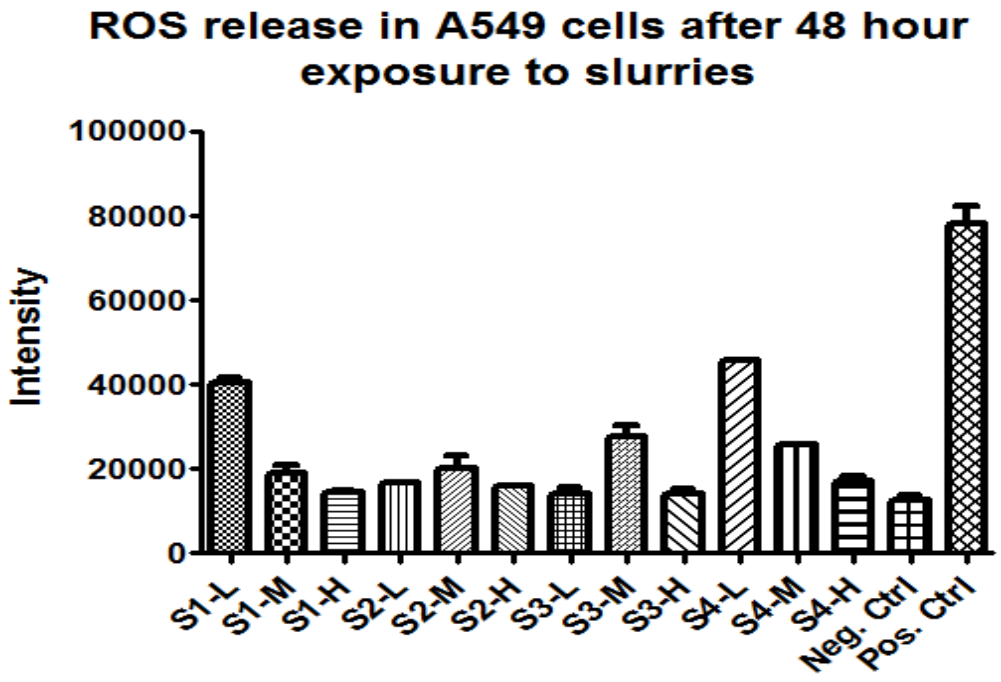
ROS release in A549 cells on exposure to slurry 3

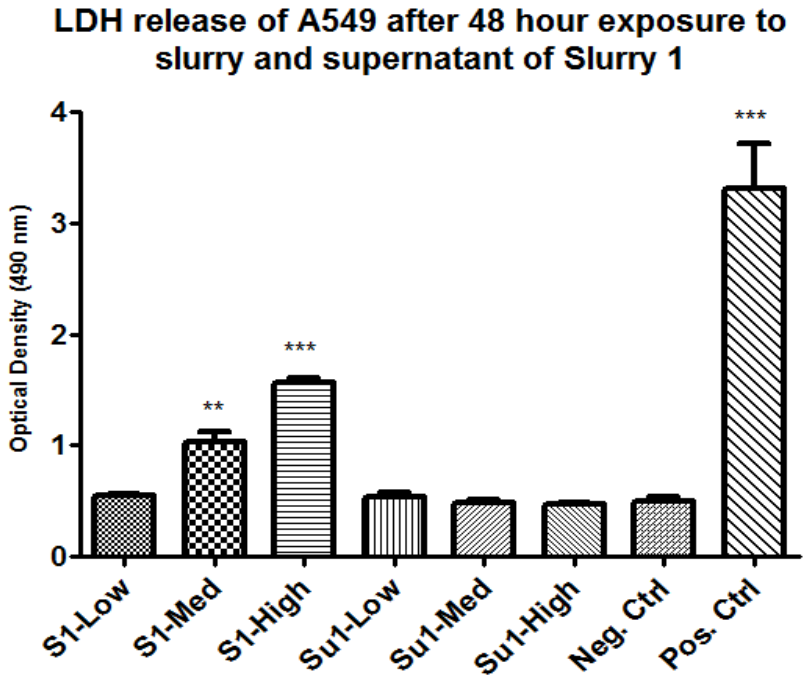


ROS release in A549 cells on exposure to slurry 4



II. Cellular Uptake and Internalization

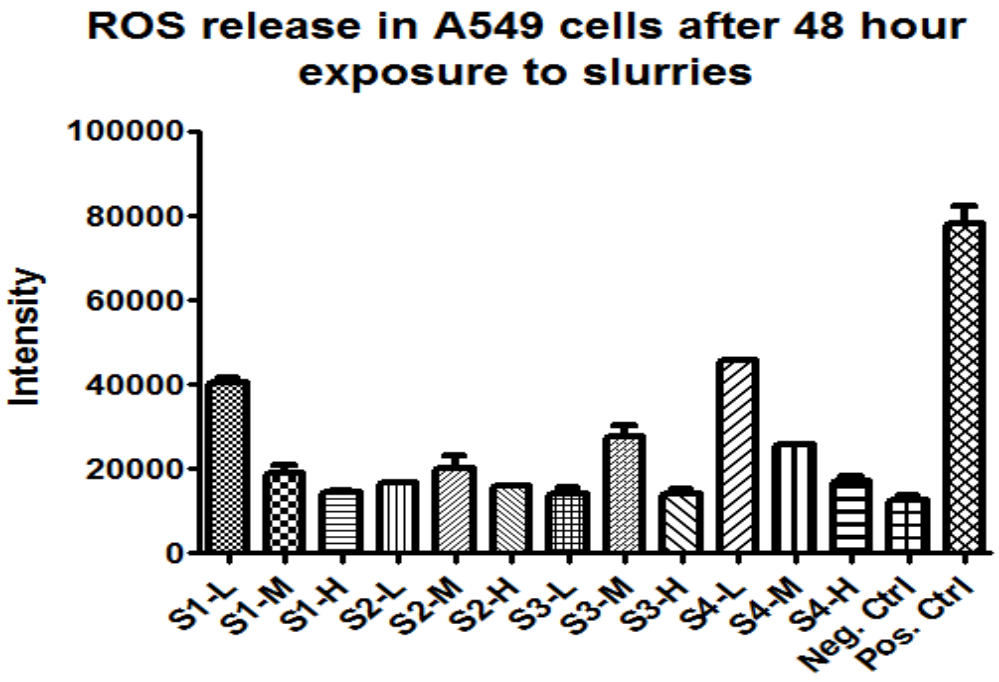


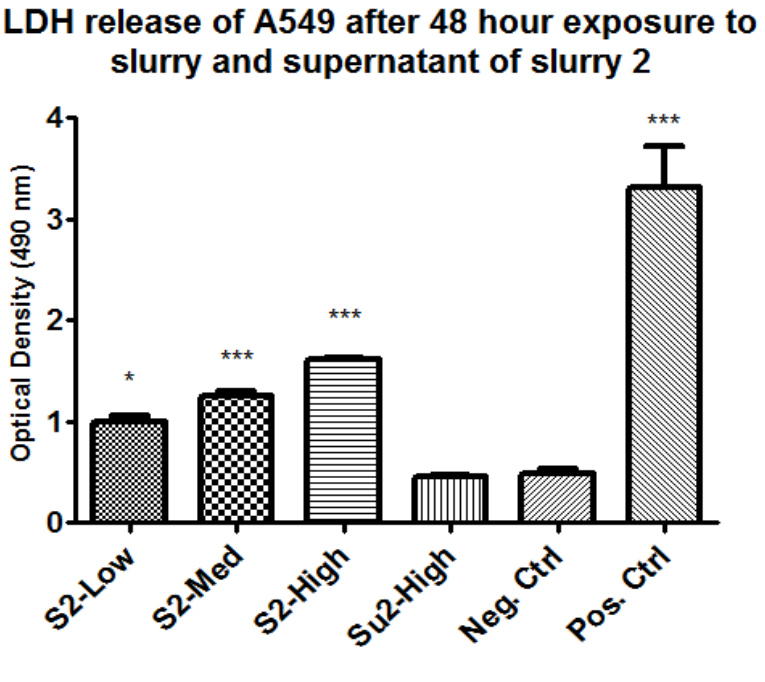


ROS Vs LDH release of slurry 1

ROS release of slurry 1 show a completely different trend when compared to LDH (LDH and MTT showed similar trends)

II. Cellular Uptake and Internalization

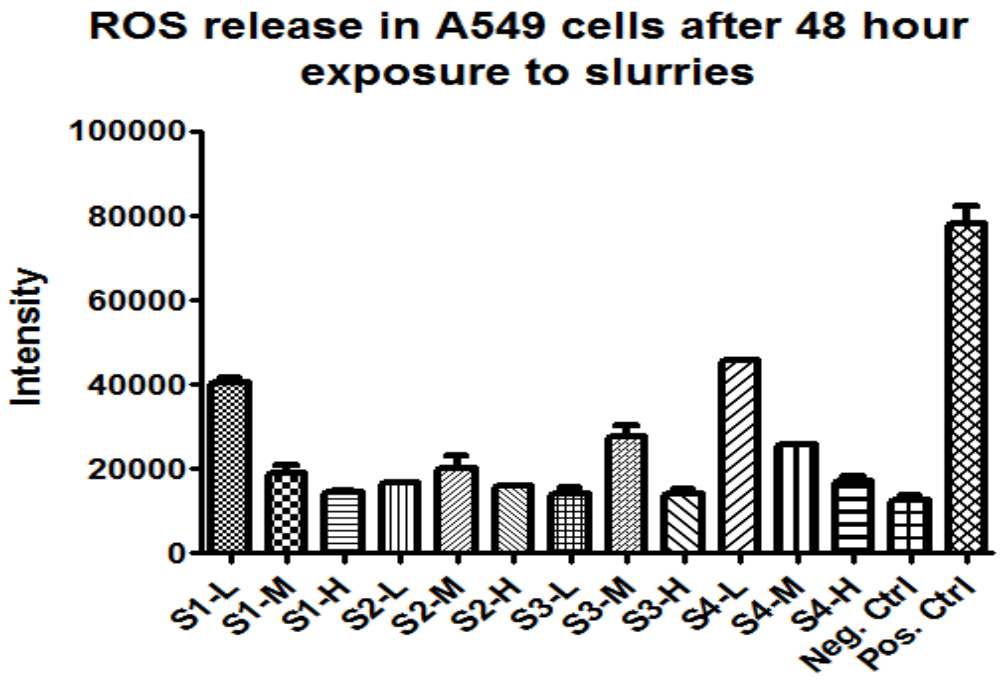


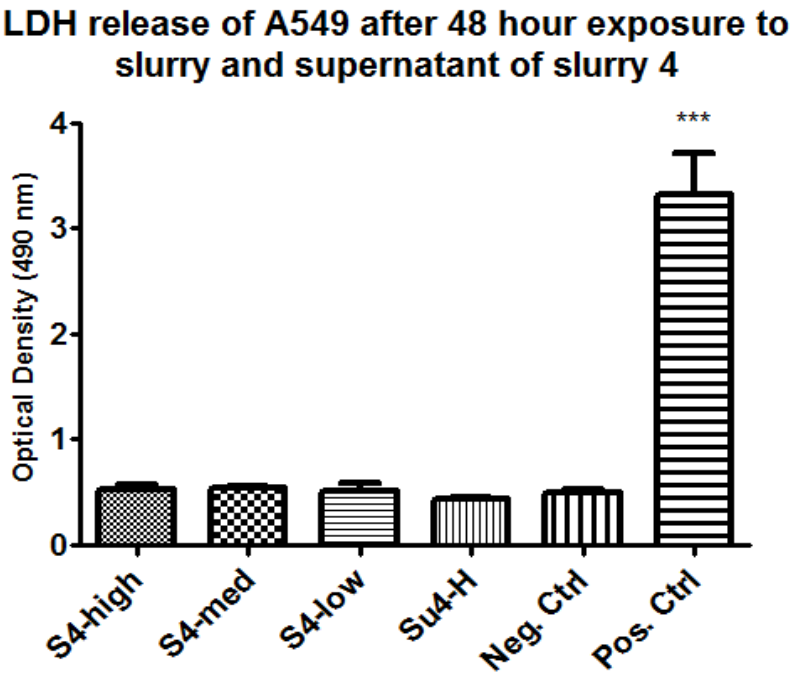


ROS Vs LDH release of slurry 2

Minimal to no ROS release is observed in cells exposed to slurry 2 but LDH release indicated different trend (LDH and MTT showed similar trends)

II. Cellular Uptake and Internalization





ROS Vs LDH release of slurry 4

High ROS release is observed in cells exposed to slurry 4 but LDH release indicated no cytotoxicity (LDH and MTT showed similar trends).

Slurry 3: No ROS, no toxocity.

Summary

- ✓ Cytotoxic effects of well dispersed slurry particles (slurry 1) showed direct relation to concentration which is not observed in particles with silica NPs which display agglomeration.
- ✓ Cellular uptake of the slurries was observed to take place when analytical tools like ICP-OES and Confocal Raman were employed.
- ✓ ECIS measurements indicate toxic effects of slurries on A549 cells.
- ✓ Preliminary ROS experiments indicated high ROS production in case of cells exposed to slurry 4 and to some extent in slurry 1 as the exposure time increases. But, slurries 3 and 4 showed minimal to no ROS.
- ✓ Trends observed in toxicity for slurries 1, 2 and 4 were observed to be completely different in ROS.

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