Task ID: 425.035

Task Title: High-Throughput Cellular-Based Toxicity Assays for Manufactured Nanoparticles and Nanostructure-Toxicity Relationships Models

Deliverable #1: Report on the validation of high-throughput cellular-based toxicity assays for MNP assessment

Summary/Abstract:

Assessment of toxicity from Manufactured Nanoparticles (MNPs) addresses ITRS grand challenge "21, Chemical and Material Assessments (ESH)". Three cell based assays carried out in 96 well plates with proper controls were validated. A549 alveolar epithelial cells were used for toxicity evaluation. Good reproducibility and low variability (% CV of ~ 10-15%) was observed with intra-day and inter-day measurements. In addition, cell uptake of MNPs was assessed qualitatively and quantitatively using Transmission Electron Microscopy (TEM) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

Technical Results and Data:

The three cell based assays evaluated were:

- **Mitochondrial Function Assay:** Evaluates the ability of mitochondria in cells to reduce a yellow dye called MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) to a blue formazan derivative which is solubilized by the addition of DMSO and absorbance measured at 570 nm. Assays were validated by using seven MNPs, (three from MNPs set 1 and four from MNPs set 2). At least 5 doses per MNP were tested with 25,000 cells/well in a 96 well plate. Intra -day and inter-day measurements were carried out by testing MNPs in at least triplicate wells per day. Therefore the reported data are means of six to eight measurements with n= 3 or 4 per day. Also controls such as MNPs with cells but no reagent were monitored to control for any interference in absorbance from inherent absorbance of MNPs. Additionally, precautions such as assessing complete solubility of formazan crystals after addition of DMSO was assessed by visual inspection under the microscope as previous reports mention interference of assay due to incomplete solubility of formazan crystals while testing toxicity of carbon nanotubes. Formazan crystals were completely solubilized after addition of DMSO in our case.

- **Reactive Oxygen Species Assay:** This assay is used to measure the ability of MNPs to induce reactive oxygen species produced in the cells. It was carried out by using carboxy H_2 DCFDA (5-(and-6)-carboxy-2´,7´-dichlorodihydrofluorescein diacetate) dye. The dye is oxidized to fluorescein by reactive oxygen species produced in the cells post treatment with MNPs and can be detected by monitoring fluorescence at 485/528 nm. Validation of this assay was done with three NPs (MNPs set 1) and ongoing experiments are evaluating ROS measurements with additional four NPs (MNPs set 2). Measurements were carried out by testing MNPs at n= 4 per well. Therefore the reported data are means of four measurements. At least 5 doses per MNP were tested with 25,000 cells/well in a 96 well plate. Controls comprised of (i) NPs without any dye to monitor for background

fluorescence and (ii) NPs with dye to monitor for fluorescence due to NP reaction with carboxy H2DCFDA in the absence of cells. A549 cells treated with 100 µM H_2O_2 were used as positive control. Untreated A549 cells without dye and with dye were used to correct for auto-fluorescence due to cells and to assess basal level of ROS in untreated cells respectively.

- **Membrane Integrity Assay:** Membrane integrity assay used neutral red dye. Neutral red is a weak cationic dye which permeates cell membrane of all cells. The retention of this dye depends on the condition of cell. Viable cells are able to accumulate this dye in lysosomes, as at acidic pH the dye is charged and gets trapped in lysosomes. In damaged cells, at end stage of cell death, the dye is no more retained in the cells. The absorbance at 540 nm measures the viable cells. Validation of this assay was done with four nanoparticles (MNPs set 2). Intra -day and inter-day measurements were carried out by testing MNPs in at least triplicate wells per day. Therefore the reported data are means of six to eight measurements with n= 3 or 4 per day. At least 5 doses per MNP were tested with 25,000 cells/well in a 96 well plate. Controls such as MNPs with cells but no reagent were monitored to control for any interference in absorbance from inherent absorbance of MNPs.

- **Details on uptake of MNPs using TEM and ICP MS** are described in Deliverable #3, "Begin to test Set 3 MNPs from ESH and SRC member companies"