Task ID: **425.024**

***Project title*:**
Environmental Safety and Health (ESH) Impacts of Emerging Nanoparticles and Byproducts from Semiconductor Manufacturing - Toxicity Assessment and Prediction

Deliverable:
Report of tests to screen the toxicity of Phase I- Nanoparticles *vs* microparticles

Background:

Numerous reports published in recent years indicate a growing concern for the potential toxicity of engineered nanomaterials (Balbus et al. 2007; Nel et al. 2006; Handy & Shaw, 2007). Toxicity research is a high priority for the semiconductor industry due to the fact that some nanoparticles (*e.g.* chemo-mechanical planarization (CMP) slurry particles) are currently used in semiconductor manufacturing, and various new nano-sized materials (nanowires, carbon nanotubes, immersion lithography nanoparticles) are being considered for upcoming manufacturing processes. Predicting the potential toxicity of emerging nanoparticles (NPs) will require hypothesis-driven research that elucidates how physicochemical parameters influence toxic effects on biological systems. Of particular concern are NPs of less than 0.1 μm that would escape normal mechanisms of cellular defense (Gwinn & Vallyathan, 2006; Stern & McNeil, 2008). The intrinsic capacity of NPs to penetrate biological tissue may in itself not be the primary cause of toxicity; rather surface properties of NPs may accentuate (or minimize) toxicity. These include high specific surface area, reactive surfaces, and adsorptive surfaces for other toxic chemicals. Contaminants can also accumulate in NPs via nano-capillary condensation (Kelvin effect) in the particle pores. NPs have very high surface curvatures, engendering high surface tensions and energies that might have unique effects on living cells. Reactive radical species can have prolonged lifetimes when sorbed onto NPs. There is a growing consensus that reactive oxygen species (ROS, composed primarily of hydroxyl radicals, hydrogen peroxide and superoxide) are a major contributing factor of NP toxicity (Gwinn & Vallyathan, 2006; Limbach et al., 2007). ROS are normally produced in and around living tissues; however, overproduction can lead to cell toxicity and loss of cell and tissue function.

Objective and key findings:

The goal of this project is toevaluate the potential toxicity of nano-materials from semiconductor manufacturing and it will elucidate the underlying toxicity mechanisms. The impact of surface contaminants and other physicochemical properties on nanoparticle toxicity will be assessed. This research will also develop new methodologies for testing and predicting ESH impacts of nanoparticles.

The goal for the first year of the project was to perform a toxicity screening of phase I nanoparticles (defined in task description as HfO_2 and SiO_2). In practice however, the toxicity screening included detailed studies of HfO_2 and CeO_2 as well as preliminary studies of several other nanoparticles (*e.g.* $Fe2O_2$, $Mn2O_3$, Fe^0, ZrO_2)

The key finding was that HfO2 and CeO2 were not found to be very toxic in any of the tests performed. Only one batch of HfO_2 caused mild toxicity, and this impact was suspected to be due to contamination rather than HfO_2 itself. CeO_2 caused mild toxicity in some assays and no toxicity in others. High toxicity was only observed for Mn_2O_3, that was also shown to generate the most reactive oxygen species. Another key finding was that dispersions of nanoparticles in general are not very stable in biological media. Therefore it is less likely they could be a toxicity concern than originally suspected.

Method of Approach

Screening of potential NP toxicity (Microtox; methanogenic toxicity; mitochondrial toxicity (MTT); and live-dead assays) will be conducted to establish a broad-based toxicity assessment. Cellular mechanisms and molecular targets of NP toxicity will be identified. A central hypothesis is that ROS are implicated. Cell-based assays will use a skin cell line and, in some cases, lung cell lines. Testing will include: *1)* ROS titers/speciation in cell-free assays with surrogate biological fluid and *in vivo* assays. *2)* Oxidative damage to biomolecules (proteins, membranes, DNA) by established chemical and/or immunochemical approaches. *3)* Mutagenicity/genotoxicity if extensive DNA oxidation occurs. *4)* Screening of gene and protein expression changes using cDNA microarrays and proteomics

Technical Results and Data:

The toxicity of three batches $HfO_2$of nanoparticles and one batch of reference micron sized HfO_2 is summarized in Figure 1 for HaCat human skin cells in the live/dead assay. The results indicate that only batch 1 HfO_2 nanoparticles displayed any noteworthy toxicity, causing 50% cell death at slightly greater than 2000 mg/L. Since this concentration is quite high, the toxicity can be considered mild. None of the other batches, including micron sized material caused any noteworthy toxicity. Batch 1 particles were not the smallest particles. Therefore no trend could be assigned to particle size. Instead it is known that batch 1 particles were made by a unique synthesis route (involving brominated compounds) and this corresponds to a unique Br fingerprint detected with the ToF SIMS technique used in Task 1 Therefore, the toxicity is suspected to be due to a unique contamination associated with the nanoparticle synthesis. All of the other toxicity assays (see Methods) provided globally speaking similar results. The only exception was that none of the batches of HfO_2 (including batch 1) were toxic to the methanogenic assay.

The toxicity of one batch of CeO_2 nanoparticles was tested in the live/dead assay as summarized in Figure 2. In this test the dispersant, dispex, was used to improve the nanoparticle dispersion (in neutral aqueous suspension, aggregates with a diameter of 1740 nm formed without the dispersant). The results indicate that properly dispersed CeO_2 caused 50% cell death between 1000 and 2,500 mg/L. Since these concentrations are quite high, the toxicity can also be considered mild. The mild toxicity was confirmed with the microtox assay as well as a new assay we are developing based on measuring O_2 uptake with yeast cells. In the methanogenic assay, $CeCO_2$ was non-toxic.

Several other compounds were screened with the microtox and yeast assay. The most toxic nanoparticle was Mn_2O_3 as shown for the results in the microtox assay which uses the chemoluminescent bacterium *Vibrio fischeri* as the target cells. As shown in Figure 3, the Mn_2O_3

nanoparticles dispersed with dispex caused 50% inhibition of bacterial growth at 60 mg/L the yeast assay (not shown), the 50% inhibition of yeast cell respiration was found to be less than 30 mg/L. These results clearly indicate that Mn_2O_3 is an acutely toxic nanoparticle. Thu the assay methods utilized can detect toxicity with nanoparticles.

One of the mechanisms hypothesized to play a role in nanoparticle toxicity is oxidative stress caused by reactive oxygen species (ROS). During the first year, extensive testing was performed to determine if the chemical reaction of nanoparticles with dissolved oxygen or with other biological molecules can cause formation of ROS. This was tested with a ROS-sensitive dye, 2′,7′-dichlorodihydrofluorescein (DCFH), which is oxidized to the fluorescent 2′,7′-dichlorofluorescin (DCF) in the presence of ROS. DCF is measured by fluorescence spectrometry. The suite of nanoparticle used in the toxicity testing were also tested for their ability to produce ROS chemically with dissolved oxygen in water alone or in the presence of the biomolecule L-dopa (a phenolic compound susceptible to oxidation). CeO_2 and Fe_2O_3 increased ROS production only in the presence of L-dopa (Figure 4A). Mn_2O_3 and Fe^0 produced ROS with water and air only (Figure 4B). The most rapid production of ROS occurred with Mn_2O_3 which was also the most toxic compounds. HfO_2 and ZrO_2 had no impact on ROS production and these compounds were the least toxic. Compounds such as CeO2 which produced ROS via reaction with other compounds (*e.g.* L-dopa) had mild toxicity. Thus a general picture is forming where chemical ROS production capacity of the nanoparticle is indicative of its toxicity. If confirmed, ROS detection with DCFH and similar dyes could be used as part of a high through-put screening program.

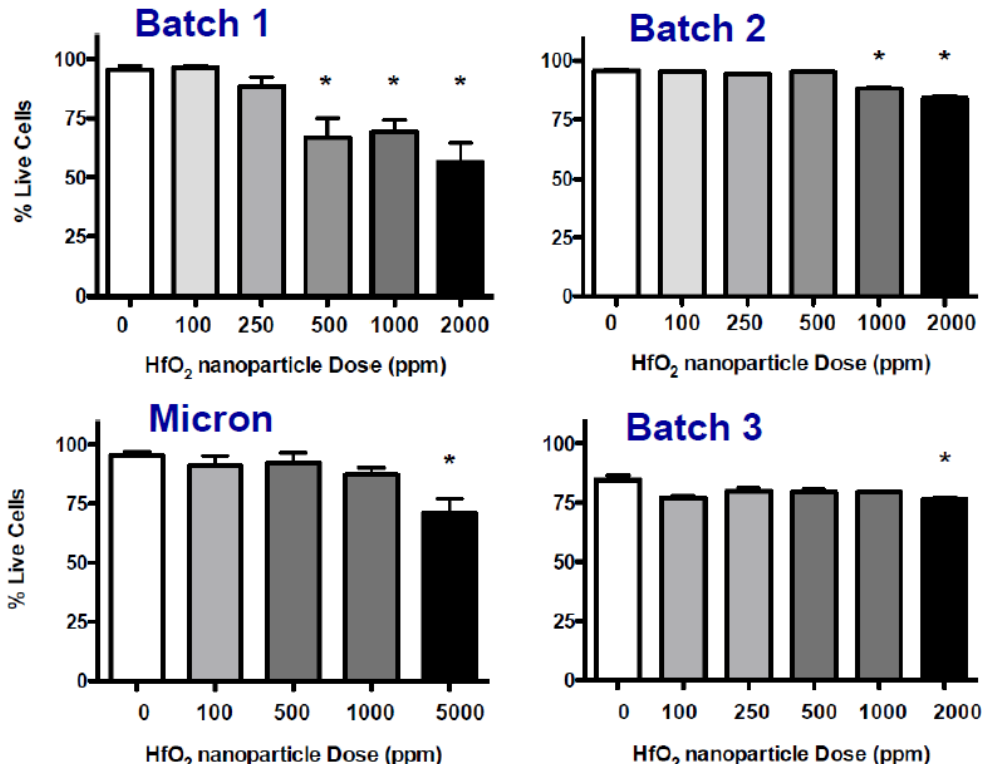

Figure 1. The toxicity of different batches of HfO_2 particles in the live/dead assay with HaCat human skin cells. The manufacture claimed average particle size was 20, 2, 100 and <44,000 nm for HfO_2 batch 1, 2, 3 and micron size, respectively. The dynamic light scattering intensity averaged particle sizes in pH < 5 aqueous suspension measured in this study was 360, 224, 169 and >6000 nm.

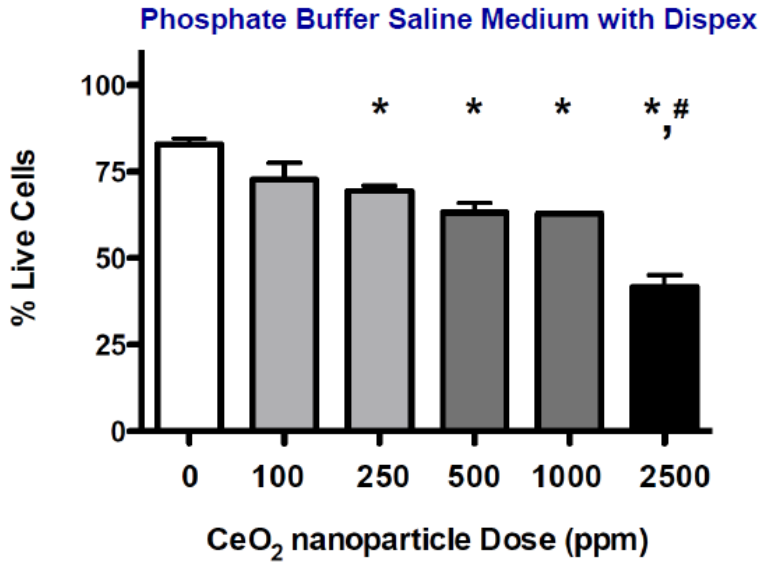

Figure 2. The toxicity of CeO_2 particles in the live/dead assay with HaCat human skin cells. The manufacture claimed average particle size was 20nmwhereas the dynamic light scattering intensity averaged particle sizes in aqueous suspension with the dispersant, dispex, measured in this study was 183 nm.

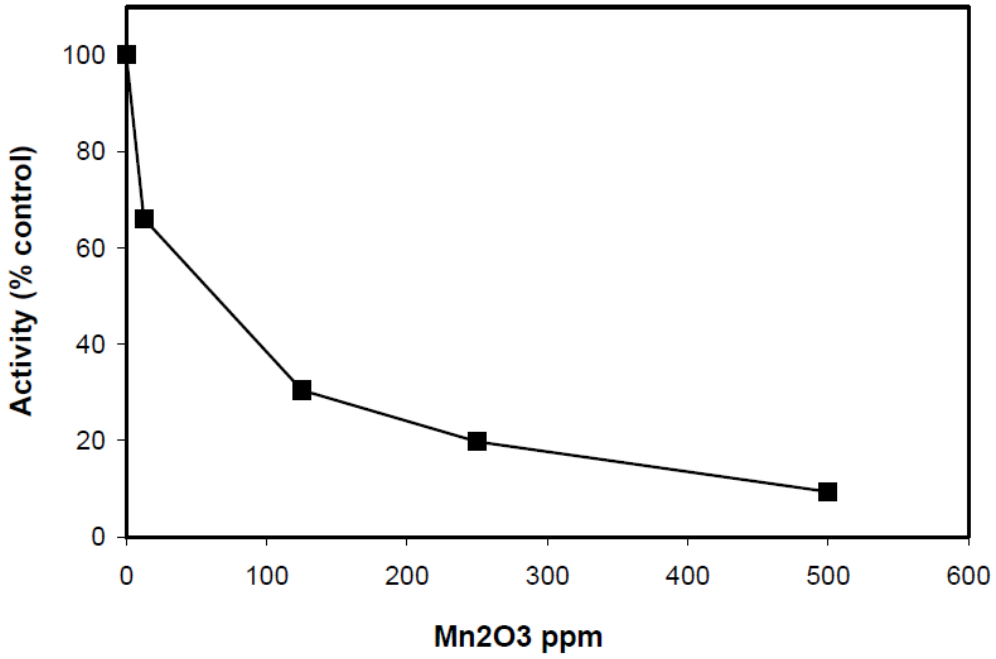

Figure 3. The toxicity of Mn_2O_3 particles in themicrotox assay with *Vibrio fischeri* bacterial cells. The manufacture claimed average particle size was 50nm.

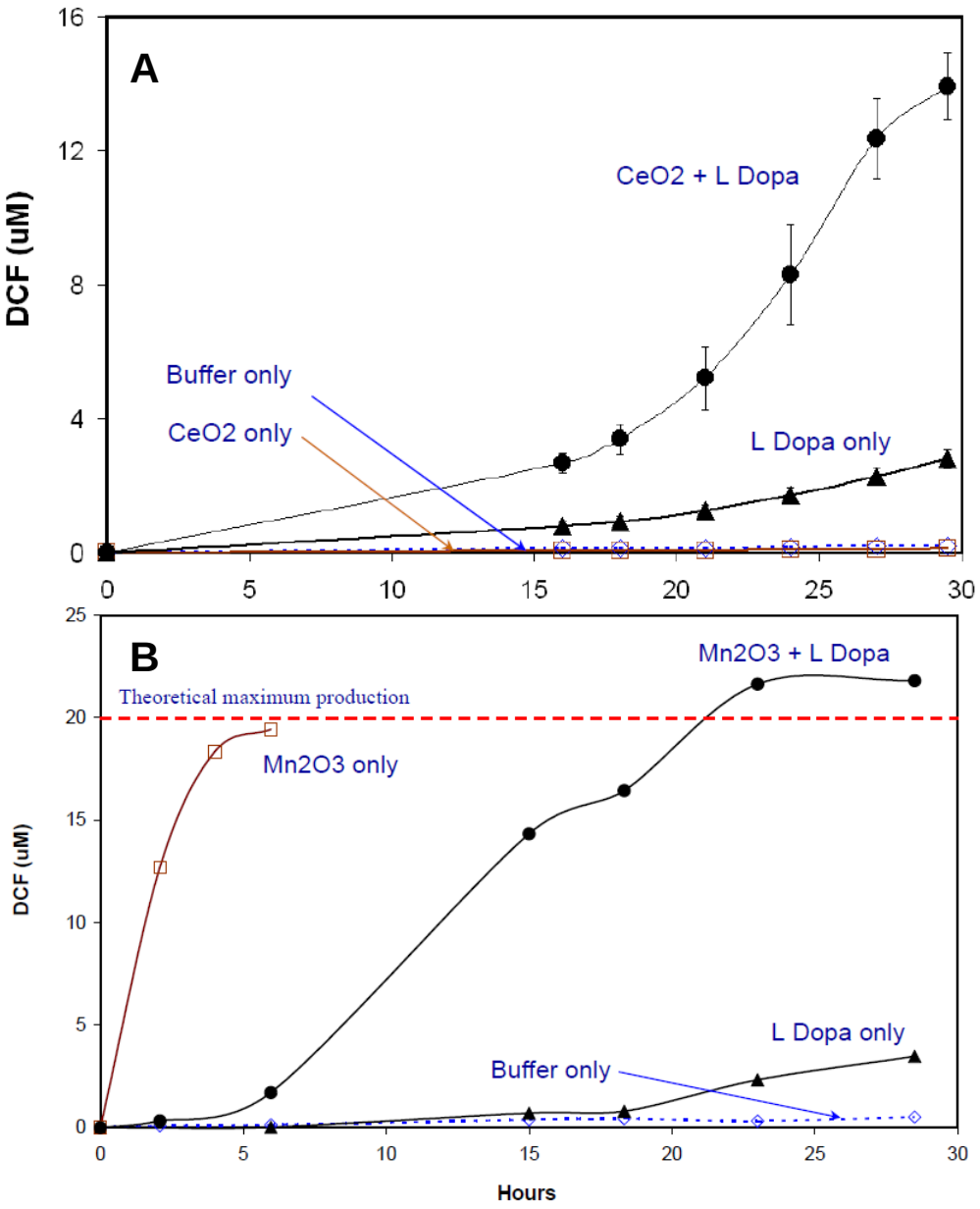

Figure 4. Formation of reactive oxygen species (ROS) as indicated by fluorescence dye (DCF) formation. **A)** Oxidation of L-dopa (3-(3,4-dihydroxypheny)l-2-amino-propionic acid) by CeO_2 nanoparticles (20 nm). The results show that ROS is produced via the oxidation of L-dopa by CeO_2 but not by direct reaction of CeO_2 with dissolved oxygen and water. **B)** Direct ROS formation during incubation of Mn_2O_3 nanoparticles (50 nm) with dissolved oxygen and water. The presence of L-dopa decreased ROS production by Mn_2O_3 (due to quenching or competitive oxidation).

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