DEVELOPMENT OF *IN-VIVO* SCREENING BENCHMARKS FOR COMPLEX ENGINEERED NANOMATERIALS

S. Mahoney[*], M. Najera[**], Q. Bai[***], E. Burton[****] and G. Veser[*****]

[*]Department of Chemical Engineering, University of Pittsburgh, slm147@pitt.edu
[**] Department of Chemical Engineering, University of Pittsburgh, mnajerita@gmail.com
[***] Department of Neurology, University of Pittsburgh, QIB1@pitt.edu
[****] Department of Neurology, University of Pittsburgh, eab25@pitt.edu
[*****]Department of Chemical Engineering, University of Pittsburgh, Pittsburgh PA 15261
gveser@pitt.edu

ABSTRACT

Complex engineered Ni-silica nanomaterials were evaluated for potential nanotoxicity risks. The materials comprised hollow and non-hollow core-shell Ni@SiO_2 and surface-deposited Ni-on-SiO_2. 5-day zebrafish embryo toxicity tests were coupled with a physiochemical characterization, including nickel ion dissolution. Zebrafish embryo survival and malformation endpoints show severe toxicity from a nickel salt which was completely mitigated in the equivalent dosing of complex engineered nanomaterials. Zebrafish motility was evaluated as a more sensitive toxicity metric and revealed a toxic effect after exposure to Ni-on-SiO_2 and hollow Ni@SiO_2, while non-hollow Ni@SiO_2 showed no toxicity effect. Correlating the toxicity results with the dissolution characteristics poses the possibility that the toxicity of these materials may be dependent upon a "Trojan horse" mechanism; hence relying on the effective take up of the nanomaterials into the zebrafish followed by nickel dissolution.

Keywords: nanotoxicity, zebrafish, engineered nanomaterials, nickel, silica

1 INTRODUCTION

Functional nanomaterials are developing at an ever accelerating pace and are finding widespread application in consumer products including cosmetics, clothing, and electronics. However, increasing evidence indicates that nanomaterials can show different toxicities as compared to their bulk components, motivating the development of methods for rapid, yet sensitive, high-throughput *in vivo* nanotoxicity screening[1,2]. Among emerging methods, zebrafish (Danio rerio) is a well-suited model due to prolific breeding and rapid development time[3] and has emerged as a robust toxicity model[4-7].

While much research is currently focused on the study of bare nanoparticles, complex engineered nanomaterials, such as embedded nanoparticles in composite materials, are much more likely to find their way into industrial practice and into consumer goods, but have received little attention in nanotoxicity studies to-date. We hypothesized that embedding metal nanoparticles (NPs) in porous silica shells – as a prototypical configuration of complex engineered nanomaterials - could reduce or entirely mitigate the metal NPs toxicity. The metal NP surface would still be accessible for catalytic reactions and hence maintaining functional properties of the embedded NP. Porous silicas are well-suited for this purpose as they constitute a versatile, easily modifiable template with low toxicity. Three different structured nickel-silica nanomaterials were investigated: Ni nanoparticles (1) embedded in hollow porous silica shells (hNi@SiO_2), (2) encapsulated in non-hollow, porous silica nanoparticles (nhNi@SiO_2), and (3) deposited on the external surface of a silica nanoparticle (Ni-SiO_2). In this work, physiochemical assessment of these nanomaterials, including dissolution, was combined with 5-day embryo zebrafish toxicity studies.

2 METHODS

2.1 Nanomaterial Synthesis and Characterization

The nanomaterials were synthesized using a one-pot, multi-step reverse microemulsion route based on a modified Stöber process. The three nanomaterials were synthesized with simple modifications to the same base process, maintaining near-identical Ni NPs sizes for all three materials as well as identical overall particle sizes. Nanomaterial particle size and morphology were characterized with transition electron microscopy (TEM, JEOL-2000FX electron microscope). Particle measurements of TEM images were done using ImageJ software. Dissolved trace metal ion concentration was measured under radial detection by ICP-AES. The degree of nickel ion dissolution from the nanoparticles in E3 media was determined at specific time points.

2.2 Zebrafish Toxicity Studies

Zebrafish embryo toxiciy studies were conducted over 5 days. The nanoparticles were dispered in E3 media (49 mM NaCl, 1.6 mM KCl, 3.3 mM $MgSO_4 \cdot 7H_2O$, pH 7.4), and added to manually de-chorionated 24 hour post fertilization (hpf) zebrafish embryos. The fish were incubated for 5 days at 28.5°C and analyzed each day visually for survival and developmental deformations. For motility analysis, surviving fish were collected on day 4, washed 3 times and replated in 96-well plates. Fish movement was captured by video on day 5. Zebrafish were exposed to a 1 hour light period followed by three 20 minute light/dark cycles. Each file was processed and quantified using MATLAB scripts as described previously[8,9]. Nickel uptake was determined by collecting the surviving zebrafish, rinsing the residual particles, digesting the fish[10], and finally measuring the nickel concentration using ICP-AES.

3 RESULTS AND DISCUSSION

3.1 Nanomaterial

TEM images of the Ni-containing nanomaterials are shown in Figure 1 and basic material characteristics are summarized in Table 1. The silica nanostructures are amorphous in all materials (as verified via X-ray diffraction), while the embedded nickel particles are crystalline. The nickel nanoparticles are similar in size (near 2 nm) and nickel content is ~9-12 wt% for all three materials. The mesoporous silica nanostructure of the materials is also similar, with BET surface areas of ~200-300 m^2/g and average pore diameters of ~0.7 nm. The primary particles are near-spherical and <60 nm.

Nanoparticle	Ni loading [wt% Ni]	D_p, Ni particle [nm]	D_p, composite particle [nm]
$hNi@SiO_2$	9.0 ±0.9	< 2	25.9 ±3.0
$nhNi@SiO_2$	9.6 ±0.4	2.5 ±0.4	46.2 ±6.5
$Ni\text{-}SiO_2$	11.9±3.2	2.1 ±0.4	56.5 ±8.2

Table 1: Ni loading and dry powder size characteristics for $hNi@SiO_2$, $nhNi@SiO_2$, and $Ni\text{-}SiO_2$ nanomaterials shown in Figure 1

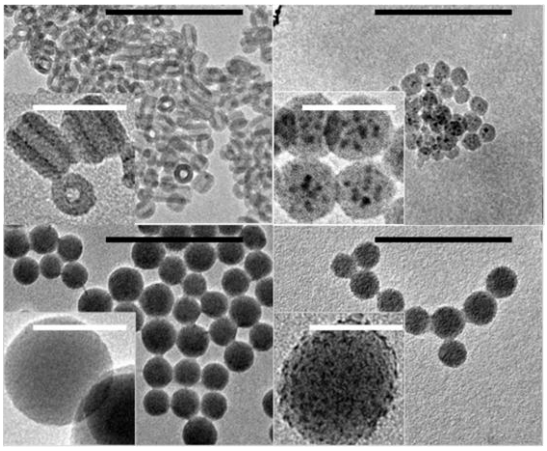

Figure 1. $hNi@SiO_2$ (a.), $nhNi@SiO_2$ (b.), metal-free SiO_2 (c.), and $Ni\text{-}SiO_2$ (d.). Scale bars are 50 nm for insets (white) and 200 nm for larger images (black).

3.2 Toxicity Studies

5-day embryo survival toxicity studies show that all three nanomaterials significantly reduce the toxicity of metal NPs compared to the equivalent dosing of an analogous metal salt ($NiCl_2$), Figure 2.

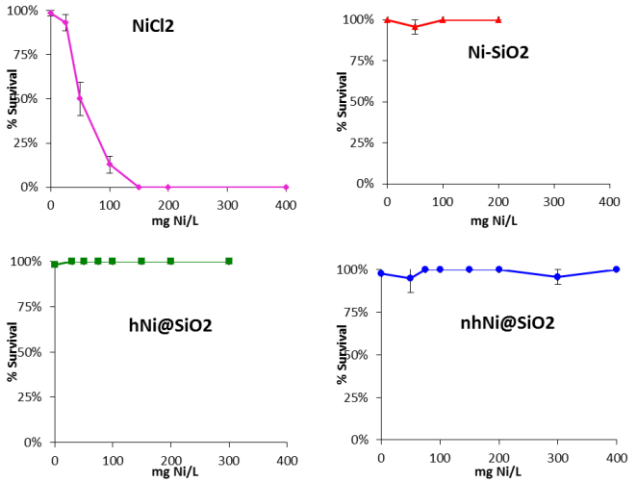

Figure 2. Zebrafish survival (%) after exposure to $NiCl_2$ or nanomaterials (as denoted in each graph) for 5 days post fertilization. Doses ranged from 5-400 mg Ni/L.

In addition to mortality, the zebrafish were monitored for malformations. Metal nanoparticles have been shown to cause a range of developmental defects in zebrafish including abnormal curvature of the vertebral column, tissue necrosis, and edema of the head, heart and gut[11,12]. Our experiments showed very few malformations upon exposure to the nanomaterials, but significant malformations after exposure to soluble nickel from $NiCl_2$ in the media (Figure 3). In agreement with the mortality results, these data demonstrate that the nanomaterials showed significantly reduced toxicity compared with free nickel ions.

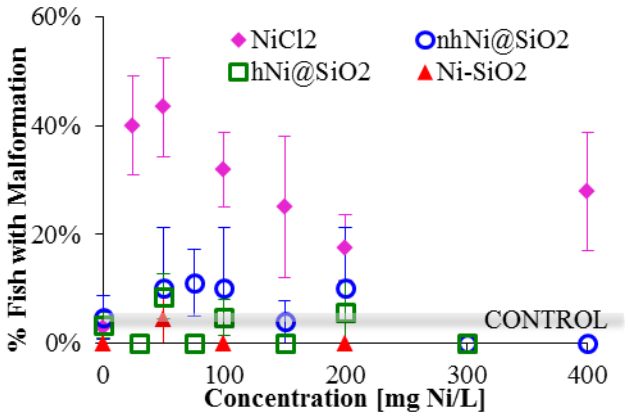

Figure 3. Malformations observed in zebrafish for $NiCl_2$ and nanomaterial over 5 days post fertilization.

Since all three engineered nanomaterials showed high zebrafish survival and low malformation rates, more sensitive means were necessary to fully evaluate the toxicity of these three nanomaterials. The studies were therefore extended to include analysis of larval motor function, a sensitive measure of larval health and nervous system function.

Figure 4 shows the mean velocity (mm/s) of larval zebrafish swimming in the wells of multiwell plates for one hour in bright light. The $NiCl_2$ positive control showed a significant higher mean velocity than the media-only and SiO_2 controls (not shown; mean velocity for E3 control was 0.93 mm/s, 2 g/L SiO_2 negative control was 1.14 ms/s and the positive 5 mg/L $NiCl_2$ control was 1.34 mm/s). The Ni-SiO_2 nanomaterial exhibited mean velocity that increased with increase in Ni concentration, while hNi@SiO2 showed a constant elevated mean velocity. In contrast, nhNi@SiO_2 showed no effect on the mean velocity compared to the media only control, indicating no apparent toxicity.

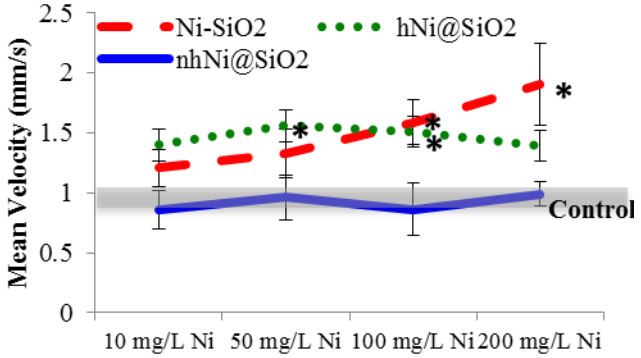

Figure 4. Zebrafish mean velocity (mm/s) for E3 control fish, hNi@SiO_2, Ni-SiO_2 and nhNi@SiO_2 nanomaterials. * indicates p>0.05 for 2-sided t-test to the E3 control, corrected with Bonferroni correction.

Overall, the zebrafish motility assay thus proved to be sensitive and allowed distinctions between the three nanomaterials over the concentration range tested: Ni-SiO_2 and hNi@SiO_2 nanomaterials exhibited an effect on motility and hence toxicity while the nhNi@SiO_2 showed no toxicity.

3.3 Dissolution Study

Dissolution of metal ions from embedded metal nanoparticles is an important characteristic to investigate since dissolution is commonly cited as an important toxicity mechanisms for metals[13], including nickel[14,15]. Figure 5 shows nickel dissolution for an initial concentration of 200 mg Ni/L in E3 media for each nanomaterial. The Ni-SiO_2 nanomaterial shows the highest Ni ion dissolution, while hNi@SiO_2 and nhNi@SiO_2 nanomaterials exhibit similar Ni ion concentrations on day 5. The different dissolution trends can be correlated well with what might be expected from the individual structures: The nickel nanoparticles in Ni-SiO_2 are fully exposed to the media solution, therefore show fast dissolution, while the Ni NPs in hNi@SiO_2 are only exposed to the hollow core, and the Ni NPs in nhNi@SiO_2 are tightly surrounded by the porous silica matrix and only exposed to the limited pore volume.

While the strong dissolution of Ni-SiO_2 could likely contribute to the significant effect on motility and be a possible source of toxicity for this material, it seems unlikely that it is the sole cause of toxicity for hNi@SiO_2 since the dissolution of hNi@SiO_2 and nhNi@SiO_2 are similar at the 5-day end point while the motility results show significant differences. We infer that the specific nanostructure must have at least a compounding effect on the toxicity of these materials.

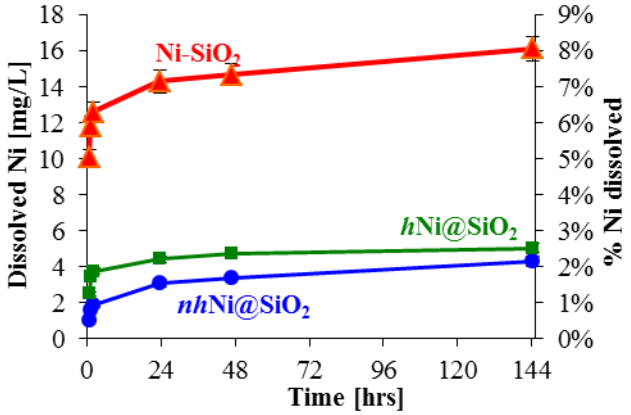

Figure 5. Amount dissolve and percent dissolution of Ni-containing nanomaterial in E3 media at room temperature for 5 days.

This conclusion is further supported by metal uptake studies: an 'uptake efficiency' index was calculated as the amount of nickel taken up by the zebrafish larva divided by the predicted amount of nickel ions available in the well from dissolution. Hence, while all nickel ions would be available in the well for nickel salts, only those ions that had dissolved from Ni NPs would be available for the nanoparticles. Figure 6 shows the uptake efficiency for each nanomaterial and the Ni salt. While the engineered

nanomaterials showed limited dissolution of metal ions into the media solution, the Ni uptake was in fact significantly enhanced when compared to the Ni salt, suggesting the presence of a "Trojan horse"[16] transport mechanism into the animal.

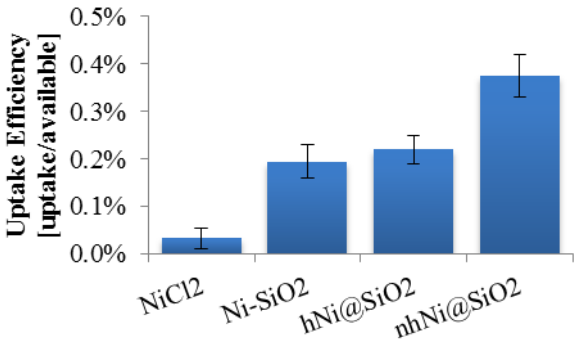

Figure 6. Uptake efficiency of NiCl2 compared to Ni-containing nanomaterials at 50 mg Ni/L exposure.

Remarkably, the nhNi@SiO_2 nanomaterial shows the highest uptake efficiency out of all three nanomaterials, yet exhibits no observable toxicity. Since this is the material with the lowest dissolution rate, we speculate that a combination of nanomaterial uptake and dissolution contributes to the observed toxicity. Thus, hNi-SiO_2 shows lower uptake and slightly increased toxicity (based on motility) due to the slight enhancement of initial dissolution rates (and thus higher integral exposure over the 5-day duration), while Ni-SiO_2 shows a comparable uptake to hNi@SiO_2 but much higher dissolution, explaining its strong toxic effect. The toxicity could hence possibly be explained by a combined effect of a Trojan horse transport mechanism of NPs into the zebrafish followed by dissolution of Ni inside the organism. Further investigations will test this hypothesis.

4 CONCLUSION

Complex engineered nanomaterials are a rapidly emerging class of nanomaterials in industrial practice and consumer products. It is therefore critical to establish rapid, high-throughput and sensitive techniques to determine the toxicity of this class of nanomaterials, and to use these toxicity assessments to develop a better understanding and ultimately structure-toxicity correlations for these materials. Zebrafish are a well-suited and established *in vivo* model that offers many different endpoints that can be utilized for nanotoxicity studies. Survival rate and developmental malformations are particularly simple and efficient endpoints to determine toxicity. Beyond those, motility offers a more sensitive functional test[17-19]. In the present studies, three carefully nanostructured nanomaterials comprised of Ni nanoparticles supported on or embedded in porous silica were tested as prototypical complex engineered nanomaterials. All three nanomaterials showed significantly lower toxicity compared to a nickel salt at the same concentration, but nhNi@SiO_2 showed no effect on zebrafish motility, suggesting that this material exhibits the lowest toxicity. Correlating the toxicity results with the dissolution characteristics indicates that the toxicity of these materials does not result from externally dissolved Ni, and that nano-specific effects may be contributing to zebrafish nanotoxicity. Direct uptake measurements allow us to hypothesize that toxicity could be the result of a "Trojan Horse" mechanism whereby physical properties of the nanoparticle enhance nickel uptake into the organism followed by internal dissolution of Ni.

Overall, these data point towards a promising and flexible way to mitigate nanomaterials toxicity, and serve to establish an experimental protocol for further studies into complex engineering nanomaterials.

5 REFERENCES

[1] Nel, A., et al., Science. **311**(5761): p. 622-627,2006.
[2] Oberdoester, G., Environmental Health Perspectives. **118**(9): p. A380-A380,2010.
[3] Nel, A., et al., Accounts of Chemical Research. **46**(3): p. 607-621,2013.
[4] Borm, P., et al., Toxicological Sciences. **90**(1): p. 23-32,2006.
[5] Dave, G. and R. Xiu, Archives of Environmental Contamination and Toxicology. **21**(1): p. 126-134,1991.
[6] George, S., et al., Acs Nano. **5**(3): p. 1805-1817,2011.
[7] Lin, S., et al., Acs Nano. **5**(9): p. 7284-7295,2011.
[8] Cario, C.L., et al., The Journal of Physiology. **589**(15): p. 3703-3708,2011.
[9] Farrell, T.C., et al., Neurobiology of disease. **44**(1): p. 9-18,2011.
[10] Borgmann, U., R. Neron, and W. Norwood, Environmental Pollution. **111**(2): p. 189-198,2001.
[11] Lee, K.J., et al., ACS nano. **1**(2): p. 133-143,2007.
[12] Scheil, V., et al., Environmental toxicology. **25**(1): p. 83-93,2010.
[13] Zhu, X., et al., Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering. **43**(3): p. 278-284,2008.
[14] Griffitt, R.J., et al., Environmental Toxicology and Chemistry. **27**(9): p. 1972-1978,2008.
[15] Ispas, C., et al., Environmental Science & Technology. **43**(16): p. 6349-6356,2009.
[16] Park, E.-J., et al., Toxicology in Vitro. **24**(3): p. 872-878,2010.
[17] Duan, J., et al., PloS one. **8**(9): p. e74606,2013.
[18] Baganz, D., G. Staaks, and C. Steinberg, Water research. **32**(3): p. 948-952,1998.
[19] Ton, C., Y. Lin, and C. Willett, Birth Defects Research Part A: Clinical and Molecular Teratology. **76**(7): p. 553-567,2006.